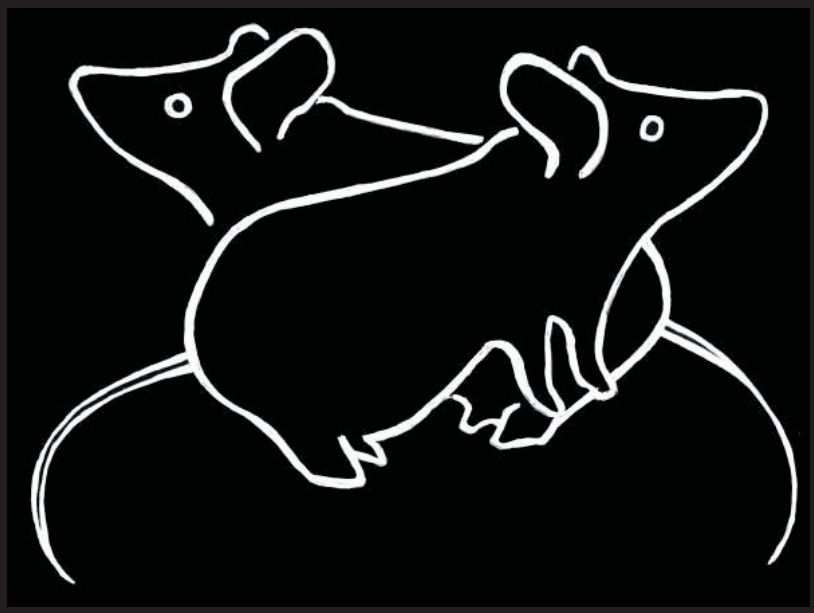
Fcγ Receptors and severe cartilage destruction in experimental arthritis



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Fcγ Receptors and severe cartilage destruction in experimental arthritis

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voor mijn ouders

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List of abbreviations

AIA	Antigen Induced Arthritis
(m)BSA	(methylated) Bovine Serum Albumin
CD	Cluster of Differentiation markers
CIA	Collagen type II Induced Arthritis
DC	Dendritic Cell
FcγR	Fcγ Receptor
GAG	Glycosaminoglycan
G-CSF	Granulocyte Colony Stimulating Factor
IC	Immune Complex
ICA	Immune complex-mediated arthritis
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
MMP	Matrix Metalloproteinase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NK	Natural Killer
PG	Proteoglycan
PLL	Poly-L-Lysine
PMN	Polymorphonuclear Neutrophil
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
Th	T helper
TNF	Tumour Necrosis Factor

Chapter 1

General introduction

Introduction

Rheumatoid arthritis (RA) is considered to be a systemic autoimmune disease, which affects about 1% of the population worldwide. RA is characterized by chronic inflammation of the synovial joints, which often results in progressive destruction of cartilage and bone. During arthritis, exacerbations and remissions occur, which lead to recurring swelling, redness, and pain in the affected joint. Histopathological features include IgG-immune complexes in synovium and articular cartilage layers and variable amounts of macrophages and T cells in the synovium, accompanied by pannus formation. Although the antigenic trigger is unknown, it is often thought that components of the articular cartilage are involved, as destructive forms of RA tend to decline when the cartilage is fully destroyed. Cartilage is a tissue with poor regenerative capacity and destruction of cartilage will eventually result in complete loss of the cartilage matrix. As the function of cartilage is to facilitate movement of joints, erosion of cartilage and bone destruction will ultimately lead to invalidity of RA patients.

Therapeutic interventions developed so far are promising, but still not able to fully prevent cartilage destruction and bone erosions. The next chapters will focus on the development of severe cartilage destruction in experimental arthritis models initiated by immune complexes and the receptors involved in the cellular interaction with IgG complexes (Fcγ receptors). Furthermore, attention is given to regulation of Fcγ receptors and its impact on the development and severity of cartilage damage.

Joint inflammation and the role of the macrophage

Diarthrodial joints are covered by synovium, which is subdivided in synovial lining layer and sublining. The synovial lining consists of macrophage-like type A cells and fibroblast-like type B cells [1]. In healthy joints, synovium has two main functions. The first is production of synovial fluid, which is essential for smooth movement of the joints [2]. The second is phagocytosis of particles present in the joint space by macrophages, hereby taking care of the first line of host defense [3,4].

The antigenic trigger in RA is not identified yet, but a characteristic event is activation of the synovial cells resulting in infiltration of inflammatory cells. The synovial lining becomes hyperplastic and is predominantly infiltrated by macrophages and lymphocytes [5-8]. The synovial joint space is infiltrated by a broad spectrum of inflammatory cells such as T cells, macrophages, B cells, polymorphonuclear neutrophils (PMNs), and monocytes [9-11]. Accumulation and activation of the inflammatory and resident cells releasing cytokines and enzymes determines the severity and prolongation of inflammation and breakdown of cartilage and bone [8,12]. It is shown that especially the macrophage has a prominent role in this process. Elegant studies have shown that the number of macrophages in joints of RA patients correlates well with joint inflammation [13] and particularly with cartilage damage [14,15]. Experimental arthritis models in the mouse have been used to extend the knowledge about the role of synovial macrophages. Macrophages can be selectively depleted from

the synovial lining and when in such a joint arthritis was induced by intra-articular deposition of immune complexes, both inflammation and cartilage damage were almost fully prevented [16-18].

Destruction of articular cartilage

Apart from the synovium, articular cartilage is also a main target tissue for destruction in RA. Cartilage covers the end of bones and is essential for smooth movement of the joints [19]. It is composed of a relatively low number of chondrocytes distributed throughout the cartilage matrix. Chondrocytes produce and maintain the extracellular matrix composed of a network of collagen fibers in which proteoglycans (PG) are entrapped [20]. These PGs are predominantly responsible for hydration, as they are highly hydrophilic by the presence of glycosaminoglycans (GAGs) [21]. All constituents of the extracellular matrix together, account for the strength and elasticity essential for normal joint function. Destruction of the cartilage matrix evolves in consecutive processes. PG depletion is the first step of degradation, and is caused by a disbalance in PG degradation by enzymes [22,23] and PG synthesis by chondrocytes [24]. PG depletion is a reversible process, which can be completely restored. When progression of cartilage destruction occurs, this leads to degradation of collagen fibers. The collagen network is difficult to repair, as collagen turnover is slow in cartilage [25] and therefore collagen damage is usually irreversible. Another characteristic of cartilage damage is chondrocyte death [26,27]. Loss of chondrocytes in the cartilage matrix might be due to an overload of destructive mediators like cytokines, oxidative metabolites, and enzymes. When chondrocytes are absent, repair of the cartilage matrix is impossible, eventually leading to erosion of the cartilage layer.

Immune complexes in rheumatoid arthritis

Activation of macrophages leading to joint inflammation and cartilage destruction can be mediated by immune complexes present in synovium, synovial fluid, serum, and even in cartilage of most RA patients [28-30]. Immune complexes are formed, by binding of specific antibodies to the antigen and the ratio of antibody to antigen determines the size and the action of the immune complex [31,32]. In RA, the most commonly used diagnostic factor is rheumatoid factor, which consists of autoantibodies directed against the Fc portion of IgG [33,34]. The antibodies themselves can consist of several classes, like IgG, IgA, or IgM [35]. More specific for RA are antibodies directed against citrullinated proteins present in RA synovium [36]. These antibodies can be detected very early in the disease and are therefore more valuable as a predictable diagnostic factor [37]. Besides RA, it has been shown that immune complexes are involved in several other diseases like SLE, glomerulonephritis, and alveolitis [38-40]. Activation of cells by immune complexes can happen via various pathways. Immune complexes are able to interact with components of the complement system, resulting in activation of the

classical [41] or alternative [42,43] complement pathway. Furthermore, immune complexes can stimulate cells via specific receptors binding immunoglobulins, resulting in release of a broad spectrum of cytokines and enzymes [44].

Receptors for IgG-containing immune complexes: Fcγ receptors

In many RA patients, IgG-containing immune complexes are abundantly present in the joint [28,34,35]. These IgG containing immune complexes can activate macrophages by binding to Fcγ receptors (FcγRs) [45-48]. These receptors recognize the Fc region of IgG, and crosslinking of these receptors results in intracellular signaling hereby triggering a wide variety of effector functions as phagocytosis, oxidative burst, and cytokine release [48,49]. Apart from the macrophage, FcγRs are expressed on other haematopoetic cells, like B cells, T cells, dendritic cells, and PMNs [50], but also on endothelial cells [51]. Three distinct classes of FcγRs have been defined: the high affinity activating FcγRI (CD64), the low affinity inhibitory FcγRII (CD32), and the low affinity activating FcγRIII (CD16) (Figure 1).

In human a total of eight genes encoding FcγRs have been identified. However, only 5 genes are functional and transcription results in expression of FcγRIA, FcγRIIA and B, and FcγRIIIA and B [47] (Figure 1, Table 1). Activating FcγRI and FcγRIIIA are associated with the common γ-chain that contains an immunoreceptor tyrosine-based activating motif (ITAM) [52-54], whereas FcγRIIA contains an ITAM motif within its molecular structure [55]. In contrast, FcγRIIB contains an immunoreceptor tyrosine-based inhibiting motif (ITIM), which inhibits ITAM-mediated cellular activation upon coligation with activating receptors [56-59]. FcγRIIIB is exclusively expressed on PMNs [46] and is not associated with either ITAM or ITIM motifs. This receptor is linked to the membrane by a glycosyl phosphatidylinositol anchor [46-48] and may func-

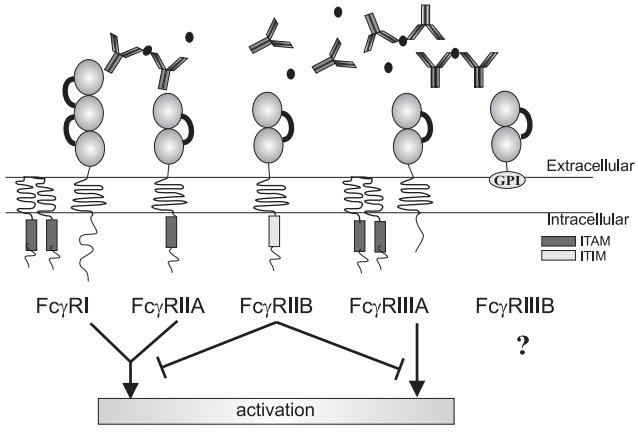

Figure 1
Schematic representation of the human leukocyte IgG Fc receptor family. Crosslinking of FcγRI, IIA or IIIA by immune complexes leads to activation of the cell. Binding of immune complexes to FcγRIIB leads to inhibition of signal transduction activated via FcγRI, IIA, and IIIA.

Table 1 General characteristics of IgG Fc receptors.

Receptor class	Receptor subclass	Distribution	IgG specificity Human	IgG specificity Mouse	Regulation Up	Regulation Down
FcγRI (CD64)	FcγRIA	monocyte, macrophage, DC[a], neutrophil[b]	3>1>4>>2[d]	2a=3>>1,2b[d]	IFN-γ G-CSF IL-10	IL-4, IL-13
FcγRII (CD32)	FcγRIIA	monocyte, macrophage, DC, neutrophil, eosinophil B cell	3>1>>2,4[d]	-		
	FcγRIIB	monocyte, macrophage, DC, neutrophil, eosinophil, B cell	3>1>4>>2[d]	2a=2b>1[d]	IL-4 IL-13	
FcγRIII (CD16)	FcγRIIIA	monocyte, macrophage, DC, neutrophil, eosinophil, NK cell[c], mast cell	1=3>4>>2[d]	1,2a,2b>>3	IFN-γ TGF-β	G-CSF TNF-α IL-4 IL-13
	FcγRIIIB	eosinophil	1=3>>>2,4[d]			

[a] DC = dendritic cell
[b] FcγRI is not expressed on murine neutrophils
[c] NK cell = natural killer cell
[d] Ligand specificity as described in reference 48

tion to facilitate FcγR signal transduction [60].

On the contrary, in the mouse, three genes encoding FcγRI, FcγRIIB, and FcγRIIIA are found, whereas FcγRIIA is not expressed [48] (Table 1). Furthermore, FcγR mediated activation of murine cells is completely dependent on ITAM structures in the common γ-chain. All three classes of FcγRs are expressed on the macrophage. Moreover, FcγRs differ in affinity for the various isotypes of IgG, like IgG1, IgG2a, IgG2b, and IgG3 [48,61] (Table 1). In the mouse, the high affinity receptor FcγRI is able to bind monomeric IgG2a, whereas IgG1 is preferentially bound by FcγRIII [62,63].

In the past years, mice deficient for one or more FcγRs have been developed, which has facilitated research regarding

the role of these receptors in immune complex-dependent diseases. Using these mice, it was shown that immune complex-mediated inflammation is determined by the coordinate expression of activating and inhibiting FcγRs [64]. Induction of a passive immune complex arthritis resulted in a tremendous rise in chronicity and joint destruction in mouse strains susceptible to collagen type II-induced arthritis (CIA) as compared to other strains. This was shown to relate to enhanced expression of activating FcγRs on their macrophages [65,66]. Furthermore, it was found that deletion of the FcγRIIB gene rendered C57Bl/6 mice susceptible for CIA, whereas normally it is difficult to induce CIA in C57Bl/6 mice [67]. Based on these studies, it was suggested that susceptibility to autoimmune arthritis is linked to disturbed FcγR expression. Recently, it was demonstrated in our lab that both FcγRII and III expression are enhanced on monocyte-derived macrophages from RA patients [68], which were hyperreactive to immune complexes as significantly increased levels of TNF-α and MMP levels were found after stimulation [68].

Mediators involved in cartilage destruction released after FcγR activation

Severe cartilage damage is characterized by chondrocyte death, matrix metalloproteinase (MMP) aggrecan degradation, and erosion of the cartilage layer (Figure 2). These parameters of irreversible cartilage damage are mainly found in experimental models in which immune complexes trigger onset of arthritis, indicating that activation of FcγRs is a crucial step in degradation of cartilage components. Moreover, we have demonstrated that severe cartilage destruction is not found when arthritis is induced in mice lacking functional activating FcγRI and III (FcR γ-chain$^{-/-}$) [69], underlining the importance of these receptors in irreversible cartilage damage.

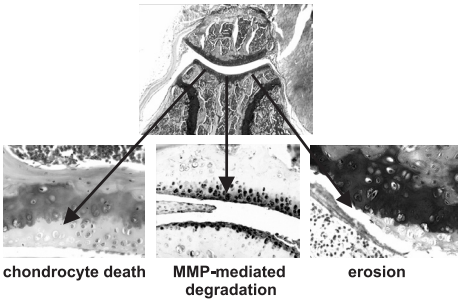

Figure 2.
Parameters of severe cartilage destruction. Irreversible cartilage damage is characterized by chondrocyte death, MMP-mediated degradation, and erosion of the cartilage surface.

Activation of macrophages via FcγRs leads to production of proinflammatory cytokines, enzymes, and oxygen radicals mediating cartilage destruction (Figure 3). Crosslinking of activating FcγRs results in production of cytokines such as TNF-α and IL-1, which are considered to be key mediators in RA [70]. Both cytokines stimulate cells to produce chemokines, which recruit inflammatory cells. Besides the prominent role in onset and continuation of inflammation, IL-1 can also generate cartilage destruction [71,72]. Intra-articular injection of IL-1 into knee joints of rabbits or mice will cause inhibition of proteoglycan synthesis in the cartilage [73-75]. In addition, IL-1 and TNF-α can stimulate chondrocytes to produce latent

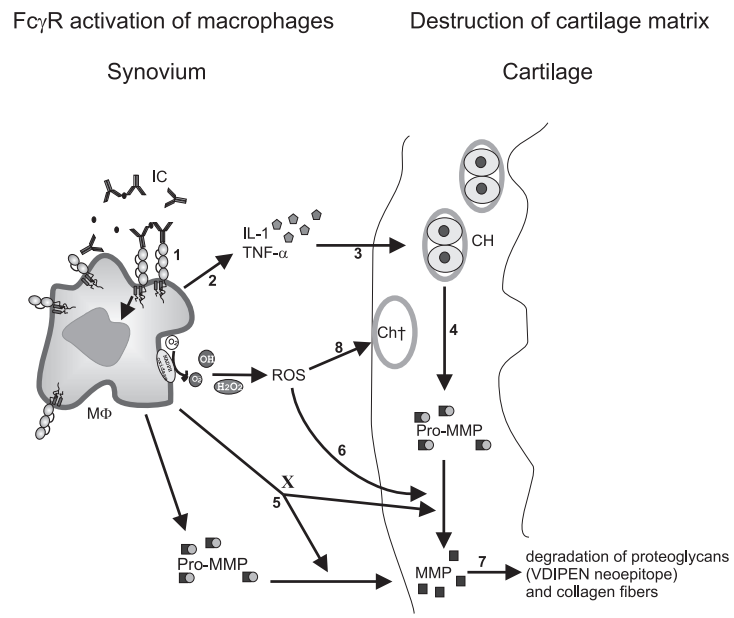

Figure 3.
After FcγR-mediated activation of macrophages (MΦ) (1), IL-1 and TNF-α are produced (2). These cytokines stimulate chondrocytes (CH) (3) to produce and release latent MMPs (pro-MMP), which accumulate in the cartilage matrix (4). The main factors responsible for activation of latent MMPs are still not identified yet (5). Reactive oxygen species (ROS) may be important candidates, since they have been shown to activate latent MMPs (6). Once pro-MMPs are activated, degradation of extracellular matrix proteins like collagen and proteoglycans occurs, eventually leading to severe cartilage destruction (7). When the macrophage is in close proximity with the cartilage surface, ROS might also induce chondrocyte death (Ch†) (8).

MMPs, which then accumulate in the cartilage matrix [76-80] (Figure 3).

Binding of immune complexes to FcγRs can also induce synthesis and secretion of latent MMPs [81] (Figure 3). In the synovium, MMPs are produced by macrophages and fibroblasts [82]. The MMP-family can be divided in four groups: stromelysins, collagenases, gelatinases, and membrane type metalloproteinases [83]. Removal of a propeptide activates the latent MMPs [84,85]. These enzymes are then capable to degrade a wide variety of substrates as gelatin, collagen, or aggrecan, but are also able to activate other MMPs [86,87]. Many MMPs have been found to cleave aggrecan at a specific site, resulting in the neoepitope VDIPEN, which remains in the cartilage. This neoepitope can be detected by a monoclonal antibody and fingerprints of MMP activity in the cartilage can be visualized [88,89] (Figure 2). Presence of the VDIPEN neoepitopes is detectable in experimental models in which immune complexes trigger arthritis onset [90,91]. The latter suggests that IC-binding to FcγRs is important in activation of latent MMPs (Figure 2). Furthermore, presence of MMPs in serum, synovial fluid, and synovium of RA patients implies involvement of these enzymes in degradation of the cartilage matrix in RA [92-94].

Additionally, binding of immune complexes to FcγRs results in release of reactive oxygen species (ROS) [95]. Oxygen radical production is mediated through activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase that is assembled at the plasma membrane [96]. ROS are capable of damaging articular cartilage either directly or indirectly [97]. Proteoglycans can be degraded by oxygen radicals as shown by in vitro studies in which the release of ^{35}S-labelled proteoglycan was increased in presence of ROS [98,99]. Superoxide-dismutase can convert ROS into hydrogen peroxide, which has a relatively long life-time and can easily penetrate through cell membranes. Hydrogen peroxide is able to inhibit proteoglycan synthesis by chondrocytes directly by reduction of intracellular ATP levels [100-102]. In addition, oxygen radicals can induce irreversible cartilage destruction by fragmentation of collagen fibers [103]. Proline and 4-hydroxyproline residues present in collagen are oxidized by ROS and become target sites for cleavage [104]. MMP-mediated cartilage destruction can also be stimulated by oxygen radicals, as on one side the natural inhibitors of MMPs are inactivated and on the other side production and activation of latent MMPs are increased [105-108] (Figure 2). Moreover, chondrocyte death can be induced, as an overload of the oxidative burst causes apoptosis of cells [109,110] (Figure 2 and 3). In addition, ROS have been demonstrated to induce increased expression of inflammatory mediators like IL-1 and TNF-α by activation of the transcription factors AP-1 and NFκB [111,112].

Modulation of FcγR expression and cartilage destruction by cytokines

Stimulation of macrophages after binding of immune complexes to FcγRs results in various mediators involved in cartilage destruction and may therefore determine severity of cartilage destruction. FcγR expression is under tight regulation of cytokines (Table 1). Granulocyte-colony stimulating factor (G-CSF) found in synovial fluid of RA patients is able to trigger a strongly increased FcγRI expression on human neutrophils [113-115]. On the contrary, FcγRIII level is strongly reduced by G-CSF [115,116], whereas no effects on FcγRII are found [117]. TNF-α selectively downregulates FcγRIII on monocytes/macrophages [118,119]. T helper 1 (Th1) and T helper 2 (Th2) cytokines are able to differentially modulate FcγR expression on monocytes. Interferon (IFN)-γ, a prototypic Th1 cytokine, induces increased expression of activating FcγRs by up-regulation of FcγRI [120-122] and FcγRIII [123]. On the contrary, the Th2 cytokine IL-4 decreases expression of FcγRI and FcγRIII, whereas levels of the inhibiting FcγRII are increased [59,124].

RA is classified as a Th1 condition since T cell clones derived from synovium of RA patients were found to produce IFN-γ [125,126]. As IFN-γ favours expression of activating FcγRI and III, stimulation with ICs may elevate cartilage destruction. On the other hand, shifting FcγR balance towards the inhibiting FcγRII with Th2 cytokines might reduce cartilage destruction. Previous studies in which IL-4 was overexpressed during CIA, demonstrated that cartilage damage indeed was de-

creased [127,128]. IL-4, however, is hardly detectable in RA synovium and synovial fluid [129]. In contrast, IL-13, which has many functions overlapping IL-4, is detected in synovial fluid and synovium of RA joints [130,131]. IL-13 is like IL-4 able to decrease expression of FcγRI and FcγRIII on human monocytes [132] and overexpression of IL-13 might therefore protect against degradation of the cartilage matrix.

Experimental arthritis models

Since RA is a complex disease involving several mechanisms, animal models are used to study the pathogenesis of chronic arthritis and to identify mechanisms underlying cartilage destruction. Furthermore, these models can be used to develop therapeutic treatments that selectively inhibit progression of destructive arthritis. Several experimental arthritis models have been developed, based on auto-antigens or foreign particles such as bacterial or yeast components that are deposited in the murine knee joint. In this thesis, the potential of, and mechanisms by which immune complexes may directly contribute to arthritis and cartilage destruction were studied using animal models in which joint inflammation is elicited by intra-articular immune complex deposition.

Antigen-induced arthritis

Antigen-induced arthritis, a T cell-dependent IC model, is based on an immune response against the foreign antigen methylated bovine serum albumin (mBSA) to which the mice are sensitized prior to induction of arthritis. Inflammation is induced 21 days after immunization by local injection of 60 μg mBSA into the knee joint. The inflammatory response is characterized by an acute phase directly after induction, followed by a chronic phase, resulting in cartilage damage characterized by chondrocyte death and erosions of the cartilage layer [133]. Joint inflammation is induced by immune complex deposition in the knee joint and propagated by antigen specific T cells.

Immune complex-mediated arthritis

Immune complex-mediated arthritis (ICA) is passively induced by systemic administration of polyclonal anti-lysozyme serum, one day before intra-articular injection of poly-L-lysine (PLL)-lysozyme. Immune complexes are formed and deposited directly in the knee joint. PLL is highly cationic and by coupling to the antigen, the formed immune complexes have an increased retention in the joint [134]. An acute inflammation develops, which reaches maximal inflammation at day 3 and wanes thereafter. Irreversible cartilage destruction can be detected 3 days after arthritis onset. In this arthritis model, no B or T cells are involved.

Aim and structure of this thesis

The aim of this thesis was to investigate the role of FcγRs in joint inflammation and cartilage destruction in experimental immune complex-mediated arthritides and to study whether the severity of cartilage damage could be modulated by regulation of FcγR expression.

First, the individual role of FcγRs in

inflammation and cartilage destruction was determined in a T cell dependent IC-mediated arthritis model (**chapter 2**). Therefore, antigen-induced arthritis (AIA) was elicited in knee joints of FcγRI-, FcγRII-, and FcγRIII-deficient mice and their wild-type controls. The results indicated that both FcγRI and FcγRIII could mediate joint inflammation, whereas irreversible cartilage damage was specifically provoked by FcγRI. The inhibiting FcγRII negatively regulated both the inflammatory response and severe cartilage destruction. In **chapter 3**, we further elucidated the mechanism by which inhibitory FcγRII reduces the arthritic response. Therefore, AIA was induced in mice lacking both activating FcγRI and III (FcγRI/III$^{-/-}$) but not FcγRII and in mice lacking all three FcγRs (FcγRI/II/III$^{-/-}$). In FcγRI/III$^{-/-}$ mice, arthritis could not be elicited, whereas in mice lacking all FcγRs, joint inflammation was tremendously increased and ICs accumulation was found in the knee joint indicating impaired endocytosis. These results suggest that FcγRII reduces joint inflammation not only by inhibition of activating FcγRI and III, but also by promoting endocytosis. Although joint inflammation was much higher in arthritic FcγRI/II/III$^{-/-}$ knee joints, severe cartilage destruction was completely prevented.

The role of FcγRs was further determined in the passively induced immune complex-mediated arthritis (ICA). In **chapter 4**, we investigated the role of FcγRs in joint inflammation, macrophage activation, and cartilage destruction during arthritis solely provoked by ICs using selective FcγR deficient mice.

The studies showed that FcγRI was critically involved in severe cartilage destruction in both AIA and ICA, as absence of FcγRI did not alter the inflammatory response, whereas severe cartilage destruction was significantly inhibited. Interestingly, in presence of T cells, irreversible cartilage destruction was more severe and completely dependent on FcγRI. This led us to speculate that T cells present in AIA might increase FcγRI expression by production of Th1 cytokines. In **chapter 5 and 6**, we investigated whether local overexpression of the Th1 cytokine IFN-γ deteriorates cartilage damage, and to what extent this process was mediated by activating FcγRs. An adenovirus encoding IFN-γ was injected into knee joints before ICA onset, and this resulted in increased severity of cartilage degradation. This effect of IFN-γ was only found in the presence of ICs, as IFN-γ overexpression during IC-independent zymosan arthritis did not induce severe cartilage destruction. These results implied a crucial role for ICs and FcγRs and this was further studied using FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice (**chapter 5 and 6** respectively). It was found that in IFN-γ-stimulated ICA, chondrocyte death was specifically FcγRI-dependent, whereas both activating FcγRI and III were able to induce MMP-mediated VDIPEN expression indicating the presence of active MMPs.

As the Th1 cytokine IFN-γ aggravated the arthritic response by up-regulation of activating FcγRs, overexpression of a Th2 cytokine might negatively regulate arthritis as these cytokines are able to decrease expression of activating FcγRs. Overexpression of the Th2 cytokine IL-13 has protective actions regarding inflammation and bone destruction in experimen-

tal arthritis. However, in these studies cartilage destruction was not investigated in detail. In **chapter 7**, the effect of IL-13 on severe cartilage damage was investigated. Local IL-13 overexpression during ICA enhanced joint inflammation. Nevertheless, chondrocyte death and MMP-mediated cartilage destruction were significantly reduced by IL-13, and were accompanied by decreased FcγRI mRNA levels in synovium.

These previous studies indicated that FcγRs are of crucial importance in the development of irreversible cartilage destruction. Moreover, a prominent role for FcγRI was found mediating chondrocyte death.

Stimulation of FcγRI leads to production of oxygen radicals via the NADPH-oxidase complex, which may induce apoptosis of cells. Furthermore, oxygen radicals have the potential to activate MMPs or inhibit their natural inhibitors. In **chapter 8**, the contribution of NADPH-oxidase driven oxygen radical production to cartilage destruction was investigated in mice lacking P47 (P47phox$^{-/-}$), which is necessary for a functional NADPH-oxidase complex. When IFN-γ-stimulated ICA was elicited in P47phox$^{-/-}$ mice, chondrocyte death and MMP-mediated proteoglycan damage were markedly diminished. Furthermore, it was found that FcγRI mRNA level was downregulated, whereas FcγRII and III were upregulated in synovium of P47phox$^{-/-}$ mice. This study showed that oxygen radicals produced via the NADPH-oxidase-pathway are critically involved in induction of irreversible cartilage damage.

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Chapter 2

Role of activatory FcγRI and FcγRIII and inhibitory FcγRII in inflammation and cartilage destruction during experimental antigen-induced arthritis

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IgG-containing immune complexes, which are found in most RA joints, communicate with hematopoietic cells using three classes of Fc receptors (FcγRI, -II, -III). In a previous study we found that if a chronic T cell-mediated antigen-induced arthritis (AIA) was elicited in knee joints of FcR γ-chain-deficient mice that lack functional FcγRI and FcγRIII, joint inflammation was comparable but severe cartilage destruction was absent. We now examined the individual role of the stimulatory FcγRI and FcγRIII and inhibitory FcγRII in inflammation and functional cartilage damage in knee joints with AIA using FcγRI-, FcγRII-, and FcγRIII-deficient mice. Three weeks after immunization with the antigen methylated bovine serum albumin (mBSA), cellular (T cell responses as measured by lymphocyte proliferation) immunity raised against mBSA was comparable in all groups examined. Humoral (total IgG, IgG1, IgG2a, and IgG2b levels) immunity against mBSA was comparable in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ but higher in FcγRII$^{-/-}$ if compared to controls. Joint swelling as measured by ^{99m}Tc uptake at days 1, 3, and 7 was similar in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice and significantly higher in FcγRII$^{-/-}$. Chronic inflammation and cartilage damage (depletion of proteoglycans, metalloproteinase (MMP)-induced neoepitopes, and matrix erosion) was studied histologically in total knee joint sections stained with hematoxylin or safranin-O. Histologically, at day 7 after AIA induction, exudate and infiltrate in the knee joint was similar in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ and significantly higher (230% and 340%) in FcγRII$^{-/-}$ mice if compared to controls. Aggrecan breakdown in cartilage caused by MMPs, which is related to severe irreversible cartilage erosion, was further studied by immunolocalization of MMP-mediated neoepitopes (VDIPEN) and image analysis. MMP-induced neoepitopes determined in various cartilage layers (tibia and femur) were primarily inhibited in FcγRI$^{-/-}$ (79 to 87% and 87 to 88%, respectively) and comparable in FcγRIII$^{-/-}$. VDIPEN neoepitopes were much higher (82% to 122% and 200% to 250%, respectively) in FcγRII$^{-/-}$ mice. Initial depletion of proteoglycans was similar (60% to 100%) in all groups. In the chronic phase, cartilage matrix erosion in the lateral and medial tibia was significantly elevated in FcγRII$^{-/-}$ (222% and 186%, respectively) but not in FcγRI$^{-/-}$ or FcγRIII$^{-/-}$ mice. These results suggest that during T cell-mediated AIA, FcγRI and FcγRIII act in concert in acute and chronic inflammation whereas FcγRI is the dominant FcR involved in severe cartilage destruction. FcγRII is a crucial inhibiting factor in acute and chronic inflammation and cartilage erosion.

Chronic inflammation and destruction of cartilage and bone are main characteristics of rheumatoid arthritis (RA) [1]. IgG-containing immune complexes (ICs), present in large amounts in joints of most RA patients have been suggested to be major pathogenic factors in RA, responsible for initiation and persistence of the inflammatory cascade and its resulting destruction of the cartilage [2]. Apart

from ICs T cells have shown to be also important in amplification of arthritis [3,4] and may enhance inflammatory reactions merely induced by ICs.

Immune complexes containing IgG, the dominant immunoglobulin in the circulation, communicate with synovial cells via cellular receptors for IgG that belong to the IgG superfamily [5-7]. Murine phagocytic effector cells express three different classes of IgG receptors (FcγRI, -II, -III) [8,9]. FcγRI and FcγRIII are heterooligomeric complexes in which ligand-binding chains are associated with the signal-transducing γ-chain. This γ-chain is required for their assembly and triggering of various effector functions including phagocytosis [10], antigen-presenting function [11], antibody-dependent cytotoxicity [12], and the release of inflammatory mediators [13]. These effector functions are regulated by an immunoreceptor tyrosine-based activation motif within the γ-chain [14].

The third receptor class for IgG, FcγRII is a single γ-chain receptor and contains an immunoreceptor tyrosine-based inhibitory motif-containing cytoplasmic domain that by co-ligation of the immunoreceptor tyrosine-based activation motif receptor inhibits cellular activation signals through the recruitment of the inositol phosphatase SHIP [15]. FcγRII has been shown to be a negative regulator of FcγRIII in IgG-IC-triggered inflammation [16].

Recently we found that activating FcγR (FcγRI and FcγRIII) were crucial in severe cartilage destruction during antigen-induced arthritis (AIA) [17]. Irreversible cartilage destruction within this model occurs through enzymatic cleavage by metalloproteinases (MMPs) of cartilage constituents. These Zn-dependent endopeptidases are capable of cleaving aggrecan and collagen type II, the main components of cartilage, which leads to severe cartilage erosion [18-20]. Various MMPs (MMP-1, -2, -3, -7, -8, -9, -13) have been found to cleave aggrecan between amino residues Asn341-Phe342 resulting in the neoepitope FVDIPEN that remains in the cartilage [21].

AIA elicited in knee joints of FcR γ-chain$^{-/-}$ lacking functional FcγRI and FcγRIII showed similar synovial inflammation if compared to controls at day 7 after arthritis induction. Nevertheless, severe cartilage destruction as MMP-mediated matrix destruction and erosion was fully absent in arthritic FcR γ-chain$^{-/-}$ knee joints. These results suggest that FcγRI and/or FcγRIII are of crucial importance in severe cartilage destruction within this model. FcγRIII has been suggested as the most likely candidate in IC-mediated joint inflammation [22].

We now investigated the involvement of activating FcγRI and FcγRIII and the inhibitory FcγRII in severe cartilage destruction seen during AIA. Expression of MMP-induced aggrecan neoepitopes and erosion of the cartilage matrix was investigated in the knee joints of arthritic mice by histology and immunolocalization. Our findings indicate that FcγRI and not FcγRIII is the dominant activatory Fc receptor involved in severe cartilage destruction in a model in which T cells play a dominant role. In contrast, FcγRII is involved in inhibition of severe cartilage destruction within this model

and may be a new therapeutic target to combat severe cartilage destruction.

Material and Methods

Animals

FcγRIII$^{-/-}$ mice were made deficient for the ligand-binding α-chain of FcγRIII (Dr. Verbeek) and were backcrossed to the C57BL/6 background for 12 generations [23]. FcγRI$^{-/-}$ were made deficient for the ligand-binding α-chain of FcγRI (Dr. Verbeek) and were backcrossed to BALB/c for 6 generations [24]. FcγRII$^{-/-}$ were developed by Dr. Takai (Sendai, Japan) [25] in the 129 SV (H-2b) and C57BL6 (H-2b) background. Control C57BL/6 and 129SV/C57BL/6 hybrids were derived from Jackson Laboratories (Bar Harbor, ME) and bred in our own facilities. Homozygous mutants and their wild-type controls, 10 to 12 weeks old, were used in the experiments.

Antibody determination in serum

Methylated BSA-specific antibodies of various isotypes (total IgG, IgG1, IgG2a, IgG2b, IgG3) were measured in sera of individual mice with an enzyme-linked immunosorbent assay (ELISA). Antigen was coated on microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) at a concentration of 100 μg/ml. Antibody titers were assessed by twofold serial dilution of the sera followed by detection of bound mouse IgG with 1:500 diluted peroxidase-conjugated rabbit anti-mouse IgG (Miles Laboratories Inc., Elkhart, IN, USA). O-Phenylenediamine (1 mg/ml; Sigma, Zwijndrecht, The Netherlands) was used as substrate for peroxidase, and the antibody titer was determined by using 50% of the maximal extinction as an end point. Sera of FcγRI$^{-/-}$, FcγRII$^{-/-}$, and FcγRIII$^{-/-}$ mice were compared to sera of their wild-type controls. In each group at least 10 mice were tested.

T cell proliferation

Mouse spleen cells were isolated and washed in RPMI supplemented with 10% fetal calf serum, glutamine (2 mM), and pyruvate (1 mM). Erythrocytes were lysed by treatment of the cells with a 0.16 M NH_4CL solution in 0.17 M Tris, pH 7.2, for 5 minutes. After two washes in RPMI, the cells were plated on plastic T flasks (75 mm^2) from Falcon Plastics, Oxnard, CA. After 60 minutes of incubation at 37°C, the nonadherent cells were harvested by aspiration and two 4 to 5 ml RPMI washes of the adherent cells. One hundred microliter of RPMI containing 1 x 10^5 T cell-enriched spleen cells were placed in each well of a sterile, U-bottomed polystyrene microculture plate (Costar, Cambridge, MA). Antigens or mitogens were added in another 100 μl to give a total volume of 200 μl, and final concentrations of antigen of 25, 12.5, and 6.25 μg/ml. Cultures were maintained at 37°C in a humidified atmosphere of 2% CO_2 for 4 days. Sixteen hours before harvesting, 1 μCi of [^{3}H]-thymidine (6.7 μCi/mmol; New England Nuclear, Boston, MA) was added in 25 μl of RPMI. Cultures were harvested with a cell harvester (Tomtec) and [^{3}H]-thymidine incorporation was quantified using a Micro Beta-plate reader (Perkin-Elmer, The Netherlands).

Induction of experimental antigen-induced arthritis

Mice were immunized with 100 μg of methylated BSA (mBSA, Sigma, Zwijndrecht, The Netherlands) emulsified in 100 μl of Freund's complete adjuvant (CFA). Injections were divided over both flanks and footpath of the forelegs. Heat-killed Bordetella pertussis (RIVM, Bilthoven, The Netherlands) was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with 50 μg of mBSA/CFA were given in the neck region 1 week after the initial immunization [26]. Two weeks after these injections, arthritis was induced by intra-articular injection of either 15 μg (FcγRII$^{-/-}$) or 60 μg (FcγRII$^{-/-}$, FcγRI$^{-/-}$, and FcγRIII$^{-/-}$) of mBSA in 6 μl of saline into the right knee joint, resulting in chronic arthritis. The approval to induce arthritis in mice was given by the local ethical committee.

^{99m}Tc Uptake measurements

Joint inflammation was measured by ^{99m}Tc pertechnetate uptake in the knee joint. This method has earlier been shown to correlate well with histological findings [27]. Briefly, mice were injected intraperitoneally with 12 μCi of ^{99m}Tc and subsequently sedated with chloralhydrate. Thirty minutes thereafter, γ-radiation was assessed by use of a collimated Na-I-scintillation crystal and the knee in a fixed position. Arthritis was scored as the ratio of the ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R:L ratios >1.1 were taken to indicate inflammation of the right knee joint.

Histology

Total knee joints were dissected, fixed in phosphate-buffered formalin (pH 7.4), decalcified in 5% buffered formic acid, and subsequently embedded in paraffin wax. Semiserial frontal whole knee joint sections (7 μm) were stained with hematoxylin and eosin (H&E) or safranin O and fastgreen. Histological parameters (joint inflammation, proteoglycan depletion, and erosion) were scored by two independent observers in a blinded manner.

Determination of proteoglycan depletion

Total knee joint sections were stained with safranin O and fastgreen. Loss of red staining from various cartilage layers (femur and tibia), which is related to loss of proteoglycans, was determined using an arbitrary scale ranging from 0 to 3. Normal cartilage and cartilage fully depleted of proteoglycans was taken as a 0 and 3 value, respectively.

Immunolocalization of MMP-induced neoepitope

For immunohistochemical analysis, sections were deparaffinized, rehydrated, and digested with chondroitinase ABC (0.25 U/ml, 0.1 M Tris-HCL, pH 8.0; Sigma) for 1 hour at 37°C, to remove chondroitin sulfate from the proteoglycans. Sections were then treated with 1% H_2O_2 in methanol for 20 minutes and subsequently 5 minutes with 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS). After incubation with 1.5% (v/v) normal goat serum for 20 minutes, sections were incubated with affinity-purified anti-VDIPEN IgG overnight at 4°C. These antibodies were kindly provided by Irwin

Singer and Ellen Bayne (Merck Research Laboratories, Rahway, NJ) and have been extensively characterized before [28,29]. In addition, sections were incubated with biotinylated goat anti-rabbit IgG and binding was detected using avidin-peroxidase staining (Elite kit; Vector Laboratories, Inc., Burlingame, CA). Induction of the peroxidase product detected using nickel enhancement and counterstaining was performed with orange G (2%) for 5 minutes.

Immunolocalization of aggrecanase-induced epitopes

Non-decalcified cryosections were digested with proteinase-free chondroitinase ABC (0.25 U/ml Tris-HCl, pH 8.0) for 1 hour at 37°C to remove chondroitin sulfate from the PGs. Subsequently, sections were fixed with periodate-lysine-paraformaldehyde fixative for 20 minutes. Sections were then treated with 1% H_2O_2 for 20 minutes followed by 5 minutes incubation with 0.1% Triton X-100 in PBS. After incubation with 1.5% normal goat serum for 20 minutes, sections were incubated for 18 hours with the primary antibody recognizing the sequence NITEGE [30]. Then sections were incubated with biotinylated goat anti-rabbit IgG and were detected using avidin-peroxidase staining. Development of the peroxidase product was done using nickel enhancement. Counterstaining was performed with orange G.

Immunolocalization of IgG

Paraffin-embedded total knee joint sections were pretreated with chondroitinase ABC and additionally stained with goat anti-mouse IgG peroxidase overnight. Development of the peroxidase product was done using diaminobenzidine (0.5 mg/ml). Sections were counterstained with H&E.

Measurement and characterization of cartilage erosion

Erosion was determined in total knee joint sections stained with H&E. Erosion was detected as ruffling of the cartilage surface and was only mild at day 7 after AIA induction. Ruffling of the cartilage surface was determined using an arbitrary scale ranging from 0 to 3. Normal cartilage surface and maximal ruffling within this experiment was taken as a 0 and 3 value, respectively.

Results

Role of the activatory FcγRIII and inhibitory FcγRII in acute and chronic joint inflammation during AIA

As the absence of a particular FcγR may alter the immunological response against methylated BSA during immunization, thereby impairing the onset and course of arthritis, we first tested cellular and humoral immunity to mBSA, 3 weeks after immunization. Cellular immunity as measured by spleen lymphocyte proliferation against mBSA showed no significant differences between knockouts and their controls (Figure 1B, D, and F). In addition, humoral immunity was measured by ELISA. Total IgG, IgG1, IgG2a, IgG2b, and IgG3 anti-mBSA levels were high but not significantly different in immunized FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ if compared to their controls (Figure 1, A and C). In sera

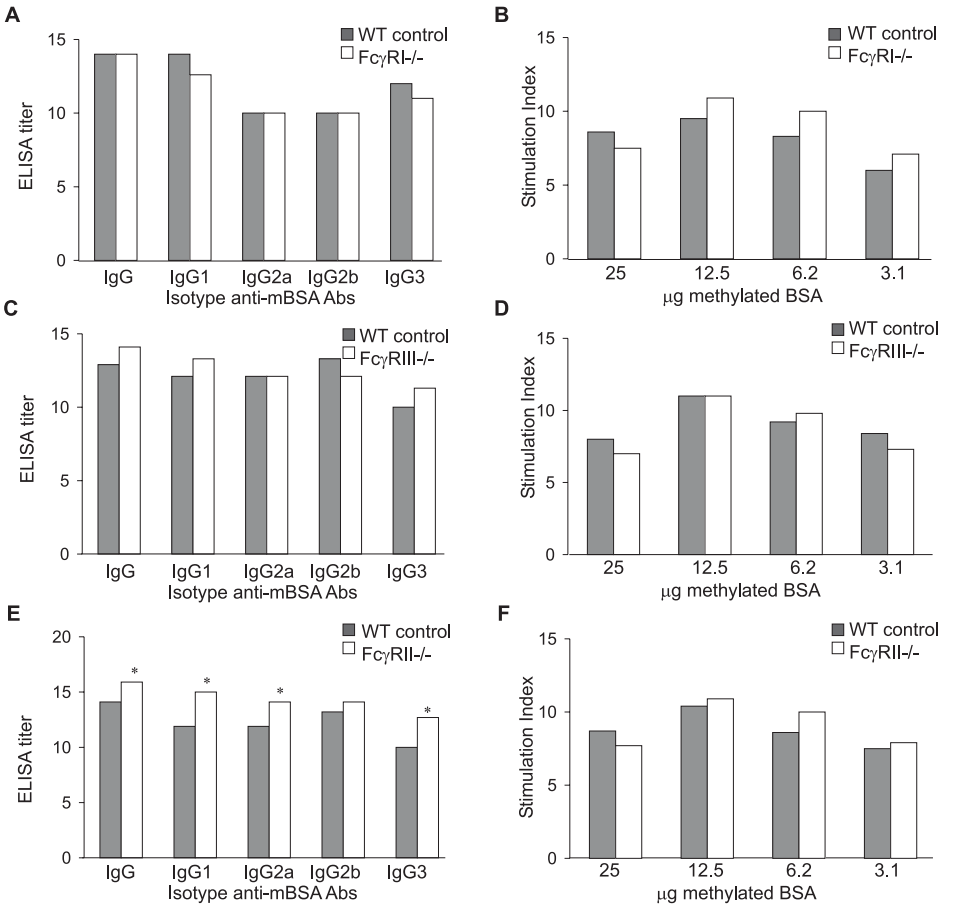

Figure 1.
Humoral and cellular immunity in FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice, and their wild-type controls was determined 7 days after induction of AIA. Humoral immunity was determined by measuring levels of various isotypes of antibodies (total IgG, IgG1, IgG2a, IgG2b) raised against mBSA using ELISA. Data of ELISA are the mean of the determination in sera of 10 mice. Mean is expressed as two-log values using 50% of the maximal extinction as an endpoint (A, C, and E). Cellular immunity was determined by measuring T cell proliferation in the presence of mBSA. ^{3}H-Thymidine incorporation was measured in cpm of spleen T lymphocytes derived from arthritic mice that were stimulated with various concentrations of mBSA (25, 12.5, 6.25, 3.1, and 0 μg/ml) (B, D, and F). The results were expressed as stimulation indexes (ratio stimulation with and without antigen) and are the mean of 8 animals. Significance was tested using the Wilcoxon rank test (*, $P < 0.05$).

of FcγRII$^{-/-}$ immunized mice however, total IgG, IgG2a, and IgG3 anti-mBSA were fourfold higher and IgG1 was even eightfold higher. IgG2b anti-mBSA was not significantly different (Figure 1E).

To investigate the role of a particular FcγR in joint inflammation, AIA was elicited by injection of 60 μg of mBSA directly into the knee joints of immunized FcγRI-, FcγRIII-, and FcγRII-deficient mice and their wild-type controls. Because FcγRIII$^{-/-}$ has been described as a main regulator of IC diseases, we expected a down-regulation of swelling in FcγRIII$^{-/-}$ mice but knee swelling was not significantly different from controls at all

time points measured (Figure 2B).

The course of knee joint swelling in FcγRI$^{-/-}$ was also comparable (Figure 2A) suggesting that FcγRI and FcγIII are redundant with respect to joint inflammation. Subsequently, we investigated the role of FcγRII in joint inflammation. Because FcγRII$^{-/-}$ mice have been shown to be highly vulnerable to ICs, injection of 60 μg of mBSA into the knee joint may be too high and for that reason we also injected a lower (15 μg) mBSA dose. Injection of 15 μg of mBSA into knee joints of FcγRII$^{-/-}$ mice resulted in a significantly higher knee joint swelling if compared to controls (2.4 versus 1.7) at day 1 but no difference was found anymore at day 4 or day 7 after AIA induction (Figure 2C). Injection of 60 μg of mBSA led to a much higher swelling at day 1 (3.0 versus 2.0) and compared to controls was still significantly higher at day 7 after AIA induction (2.0 versus 1.1) (Figure 2D).

In addition, cellular infiltration and exudate in the knee joint was studied by histology. At day 7 after induction of AIA, total knee joint sections were made and stained with hematoxylin and eosin. In FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ arthritic knees, similar exudate and infiltrate was measured in all animals studied (Figure 3, A and B). In arthritic knees of FcγRII-deficient mice, both exudate and infiltrate were found to be significantly elevated (180% and 242% in the 60-μg group, respectively) (Figure 3D). This reached significance only in mice injected with the high dose of mBSA (Figure 3D, E, and F). As the total number of inflammatory cells present in the synovium at day 7 after AIA was not different in

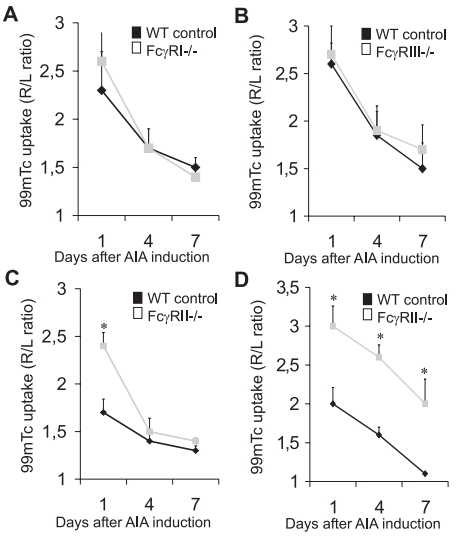

Figure 2.
R:L ratios of ^{99m}Tc uptake at various days (1, 4, and 7) after intra-articular injection of mBSA in knee joints of mBSA-immunized FcγRI$^{-/-}$ (A), FcγRIII$^{-/-}$ (B), and FcγRII$^{-/-}$ (C and D) mice, and their wild-type controls. In knees of FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice, 60 μg of mBSA was injected (A and B) and in knees of FcγRII$^{-/-}$ mice, 15 or 60 μg of mBSA (C and D) was injected. Values represent the mean ± SD of 10 mice. Data were evaluated using the Wilcoxon rank test (*, $P < 0.05$). Note the significantly higher joint swelling in FcγRII$^{-/-}$ mice, after injection of 15 μg of mBSA at day 1(C) and after injection of 60 μg of mBSA at days 1, 4, and 7 (D).

FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ and higher in FcγRII$^{-/-}$ mice, we further investigated whether the absence of a particular Fc receptor might influence the type of inflammatory cell present in the joint. The polymorphonuclear leukocyte/macrophage ratio was determined by immunolocalization using NIMP-R14 that stains polymorphonuclear leukocyte specifically. In arthritic knee joints of FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice and their controls, no differences were found in the polymorphonuclear leukocyte/macrophage ratio that was 40:60 in the exudate and 25:75 in the infiltrate (data not shown).

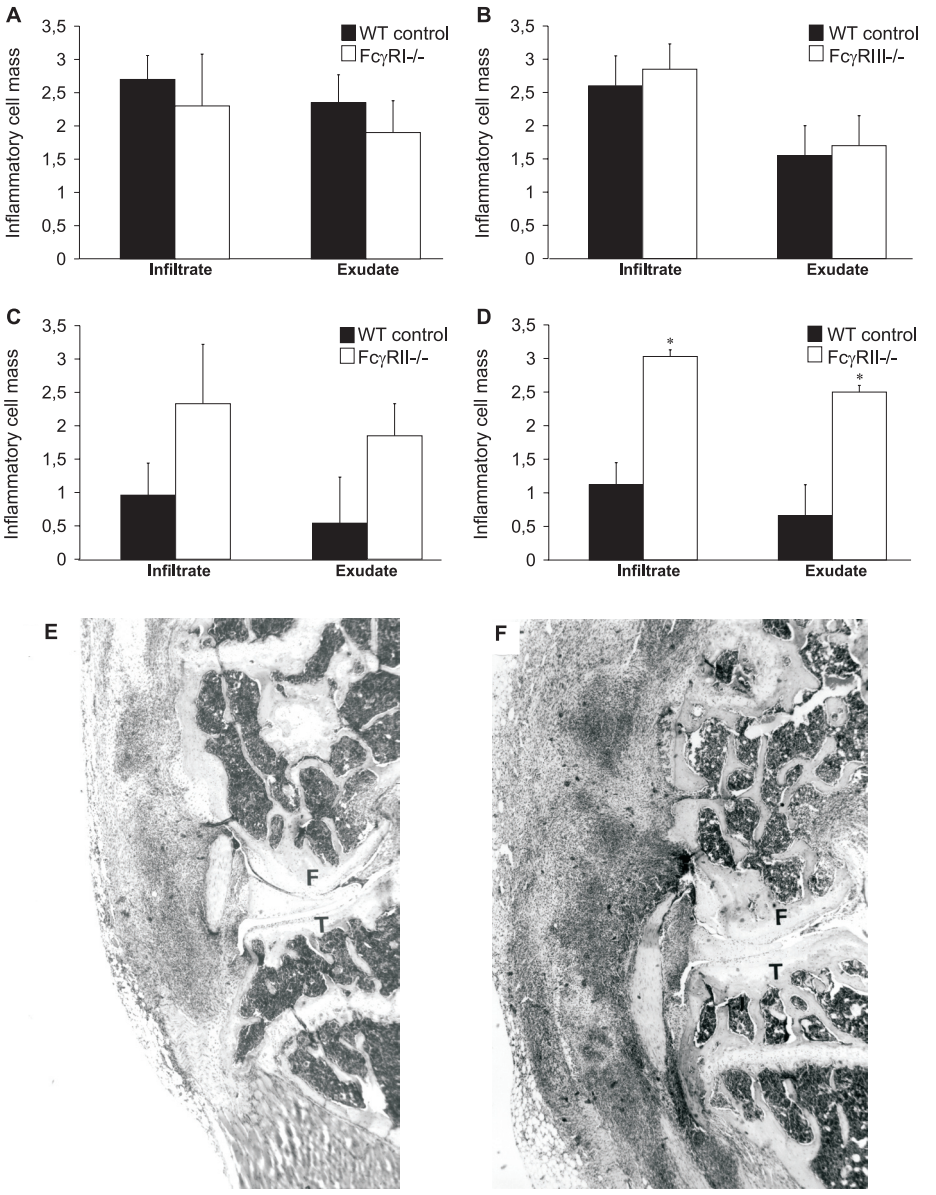

Figure 3.

Frontal sections of whole knee joints 7 days after induction of AIA in FcγRI$^{-/-}$ (A), FcγRIII$^{-/-}$ (B), FcγRII$^{-/-}$ 15 μg (C), and FcγRII$^{-/-}$ 60 μg (D) mice, and their wild-type controls. The amount of cells present in the synovium (infiltrate) and in the joint cavity (exudate) was determined using an arbitrary scale from 0 to 3. 0, No cells; 1, minor; 2, moderate; 3, maximal. The amount of cells was determined by two blind observers. Data are the mean ± SD of 10 animals. Significance was tested using the Wilcoxon rank test (*, $P < 0.05$). Original magnifications, x100. F, Femur; T, tibia. Note the significantly higher infiltrate and exudate at day 7 after injection of 60 μg of mBSA in knee joints of FcγRII$^{-/-}$ (D and photographs F = FcγRII$^{-/-}$ versus E = wild-type control) and comparable cell mass in arthritic FcγRI$^{-/-}$ (A) and FcγRIII$^{-/-}$ (B) mice.

As Fc receptors have been shown to be involved in removal of ICs from various body compartments, the presence of IgG ICs localized in the arthritic joints at day 7 AIA was determined using rabbit anti-murine IgG antibodies. No significant differences in IC deposition were found in the knee joints of FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice and their controls (data not shown).

Activatory FcγRI and FcγRIII and inhibitory FcγRII are not involved or redundant in loss of proteoglycans from cartilage layers in knee joints with AIA

Subsequently, we studied the role of FcγRI, FcγRIII, and FcγRII in cartilage damage. The earliest cartilage damage seen during experimental arthritis is loss of proteoglycans from the cartilage matrix that is evident between 24 and 48 hours after AIA induction. Proteoglycan breakdown was measured by determining the loss of red staining in safranin O-stained knee joint sections using an arbitrary scale from 0 to 3. At day 7 after induction of AIA, loss of red staining in the cartilage layers of femur and tibia in control mice injected with 60 μg of mBSA reached almost maximal values (Figure 4 A, B, and D). In arthritic knee joints of FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice (Figure 4A, B, and D, respectively), proteoglycan depletion was not significantly different from their arthritic control groups. Injection of the 15 μg mBSA dose showed lower PG depletion (Figure 4C). Although proteoglycan depletion was slightly higher in the FcγRII$^{-/-}$ mice, this difference did not reach significance.

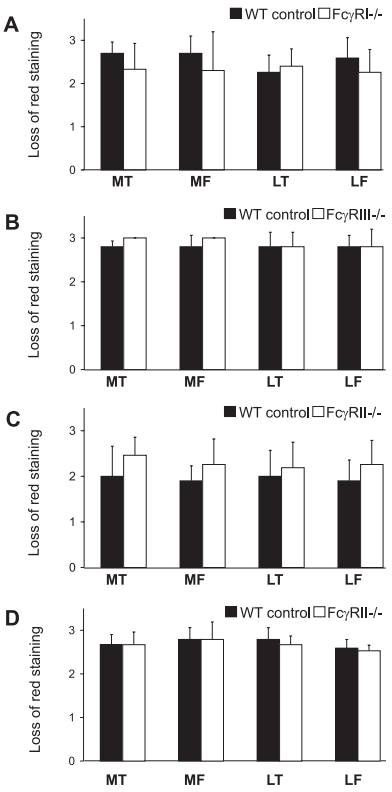

Figure 4.
Loss of red staining from cartilage layers of total knee joint sections 7 days after induction of AIA in FcγRI$^{-/-}$ (A), FcγRIII$^{-/-}$ (B), FcγRII$^{-/-}$ 15 μg (C), and FcγRII$^{-/-}$ 60 μg (D) mice. Loss of red staining was scored in tibia and femur using an arbitrary scale from 0 to 3. Data are expressed as loss of red staining if compared to control cartilage layers and represent the mean ± SD of 10 mice and were tested on significance using the Wilcoxon rank test (*, $P < 0.05$). No significant difference in PG loss was found between wild-type controls and FcγRI$^{-/-}$ (A), FcγRIII$^{-/-}$ (B), and FcγRII$^{-/-}$ (C and D) mice. MT, Medial tibia; MF, medial femur; LT, lateral tibia; LF, lateral femur; F, femur; T, tibia. Original magnifications, x100.

FcγRI activates, whereas FcγRII inhibits the induction of MMP cleavage site neoepitope VDIPEN during AIA. No effect of FcγRIII on VDIPEN expression

Within AIA, MMPs have been shown to be involved in degradation of aggrecan [31,32] and collagen [33] leading to irreversible cartilage destruction. MMPs degrade aggrecan leaving the C-termi-

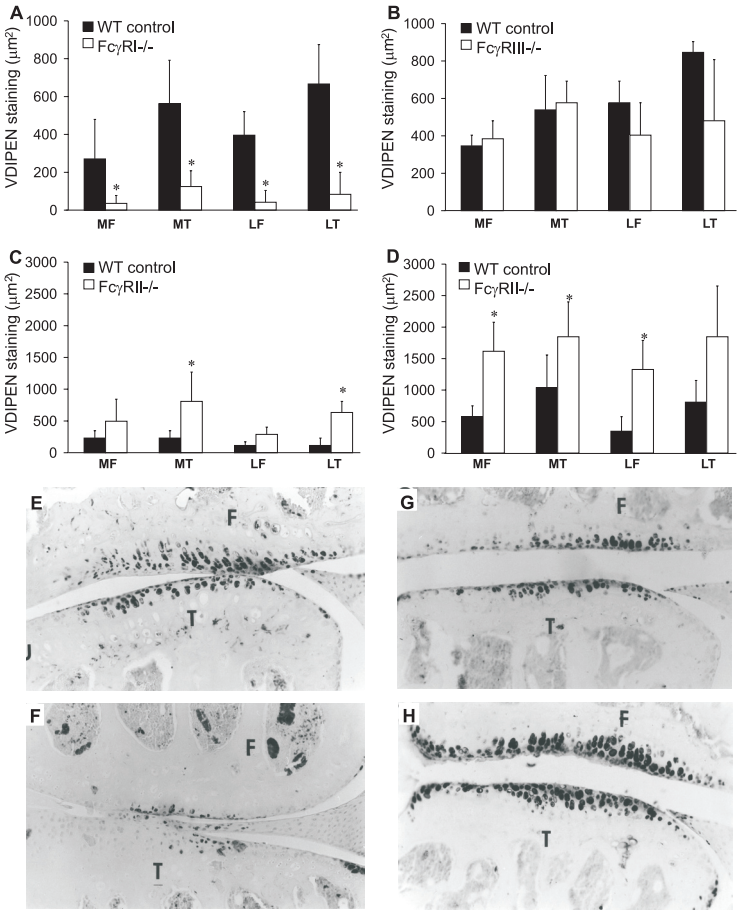

Figure 5.
Expression of VDIPEN staining in knee joints of FcγRI$^{-/-}$ (A), FcγRIII$^{-/-}$ (B), FcγRII$^{-/-}$ 15 μg (C), and FcγRII$^{-/-}$ 60 μg (D) mice, and their wild-type controls 7 days after AIA induction. Positive VDIPEN staining was determined in various cartilage layers at an original magnification of x100 (MT, medial tibia; MF, medial femur; LT, lateral tibia; LF, lateral femur; F, femur; T, tibia) using automated image analysis and expressed in VDIPEN staining per μm² cartilage. VDIPEN expression was significantly lower in cartilage layers of arthritic knee joints of FcγRI$^{-/-}$ (A and micrographs F = FcγRI$^{-/-}$ versus wild-type control = E) but not in FcγRIII$^{-/-}$ mice if compared to wild-type controls (B). VDIPEN expression was elevated in arthritic knee joints of FcγRII$^{-/-}$ (C and D). Difference reached significance in the lateral and medial tibia after injection of 15 μg of mBSA (C) and in the lateral and medial tibia and medial femur after injection of 60 μg of mBSA (D and photographs H = FcγRII$^{-/-}$ versus wild-type control = G). Data represent the mean ± SD of 10 mice. Original magnifications, x250.

nal ending with the amino acid sequence VDIPEN that can be detected by specific antibodies around day 5 after induction of AIA [32]. For this reason, AIA day 7 was taken to detect VDIPEN expression in the cartilage matrix.

The amount of VDIPEN was measured by determining the area of cartilage expressing VDIPEN using automated image analysis. In all investigated knee joints of wild-type mice injected with 60 μg of mBSA, prominent VDIPEN staining was found in the cartilage layers of tibia and femur (Figure 5 A to D). Strikingly,

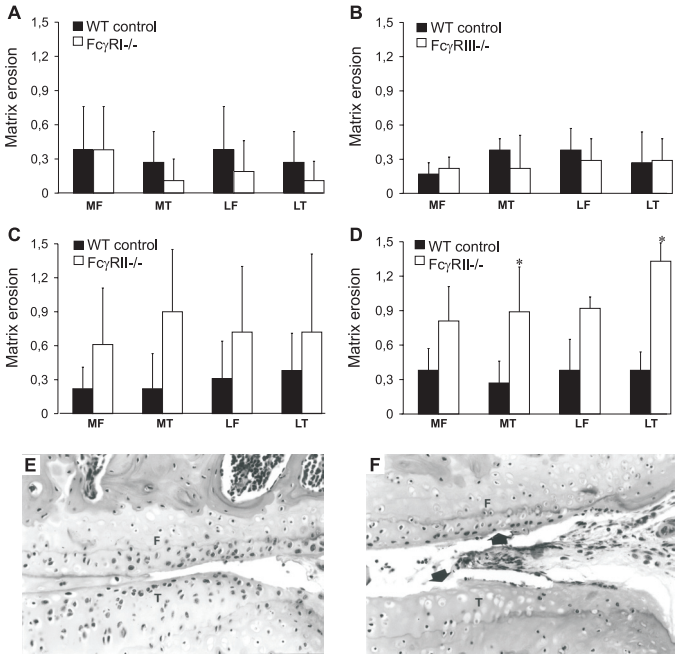

Figure 6.
Erosion in cartilage layers (LF, lateral femur; LT, lateral tibia; MF, medial femur; MT, medial tibia) of arthritic knee joints at day 7 after AIA induction in FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice, and their wild-type controls. Erosion of the cartilage surface was defined as ruffling of the cartilage surfaces using an arbitrary scale from 0 to 3. In FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice, erosion was mild and comparable to wild-type controls. Erosion was found in 8 of 10 animals in both groups (A and B). Injecting 60 μg of mBSA in knees of FcγRII$^{-/-}$ resulted in elevated erosion in all cartilage layers that reached significance in the lateral and medial tibia (D and F: FcγRII$^{-/-}$ and their wild-type control, E). After injection of 15 μg of mBSA in knee joints of FcγRII$^{-/-}$, erosion was higher but did not reach significance (C). Original magnifications, x100.

in arthritic knees of FcγRI$^{-/-}$, VDIPEN expression was significantly lower in medial and lateral femur (88% and 87%, respectively) and in medial and lateral tibia (79% and 87%, respectively) (Figure 5A and micrographs in Figure 5, E and F). In arthritic knees of FcγRIII$^{-/-}$, VDIPEN expression was comparable to controls (Figure 5B). In contrast, in FcγRII$^{-/-}$ arthritic joints, VDIPEN was significantly higher in lateral and medial femur and lateral tibia (250%, 200%, and 122% higher, respectively), indicating that FcγRII is involved in MMP-mediated cartilage destruction (Figure 5, C and D and micrographs in Figure 5, G and H). VDIPEN expression was much lower (between 56 to 76%) when 15 μg of mBSA was injected if compared to the 60 μg group but still significantly higher in lateral and medial tibia of FcγRII$^{-/-}$ if compared to their wild-type controls (Figure 5C).

FcγRII is involved in inhibition of erosion of the cartilage matrix

To examine the role of FcγRI, FcγRIII, and FcγRII in severe irreversible cartilage destruction, we also analyzed erosion of the cartilage matrix in paraffin sections. Erosion of the cartilage matrix that is only mild at day 7 after AIA was determined as ruffling of the cartilage surface. No sig-

nificant differences in matrix erosion were found in knees of FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice if compared to their wild-type controls (Figure 6, A and B). In contrast, in knee joints of FcγRII$^{-/-}$ mice, erosion was enhanced in the 60 μg mBSA group and this difference reached significance in the lateral and medial tibia (222% and 186% higher, respectively) (Figure 6D and micrographs in Figure 6, E and F). In the 15 μg mBSA group, although erosion was somewhat higher, differences did not reach significance (Figure 6C).

Discussion

In a previous study, we found that absence of functional FcγRI and FcγRIII is related to acute and sustained inflammation and is also a major determinant of severe destruction of cartilage in AIA. In the present study we show that both FcγRI and FcγRIII are important in synovial inflammation, whereas FcγRI is the dominant activatory receptor involved in severe cartilage destruction within this model. In contrast, FcγRII is crucial in negative regulation of both acute and chronic inflammation and late severe cartilage destruction.

Because the absence of a particular FcγR may impair the T and B cell-mediated immunological response against the arthritogen mBSA, cellular and humoral immunity against mBSA was determined. T cell responses against mBSA were however not different in immunized FcγRI$^{-/-}$, FcγRIII$^{-/}$, and FcγRII$^{-/-}$ mice and their controls and is in line with earlier studies showing that loss of functional FcγRI and FcγRIII did not perturb T-cell maturation [34]. FcγRIII is also expressed on mast cells, and FcγRIII-deficient mice lack IgG-mediated mast cell degranulation [35]. However, earlier studies by our laboratory showed that the course of AIA elicited in knee joints of mast cell-deficient (WBB6F1-W/Wv) mice was not significantly different from control littermates, suggesting that mast cells do not play an important role in joint inflammation within this model [36].

Measuring humoral immunity, isotype-specific antibody responses against mBSA were not different in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$, which is in line with earlier studies [37]. In immunized FcγRII$^{-/-}$ however, all anti-mBSA isotype antibody titers (with the exception of IgG2b) were four-fold to eight-fold elevated if compared to controls. In the present study, we investigated FcγRII$^{-/-}$ on a hybrid SV129/C57BL6 background, because this background does not display abnormalities in the immunological status. FcγRII$^{-/-}$ mice on other backgrounds (eg, C57BL/6) develop autoantibodies of multiple IgG subclasses [38] that might enhance the arthritis induced by mBSA immunization. Differences in autoimmune development however suggest that FcγRII in combination with genetic loci may be important in breaking the tolerance not only against an autoantigen such as collagen type IV [39] but also against an exogenous antigen such as mBSA. FcγRII may function to maintain tolerance in the periphery and together with genetic loci, gate B cell activation against various antigens.

As the adaptive immunity in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ is not altered, local expression of both activating FcγR in the joint may drive synovial inflammation and cartilage destruction. Immune complex-

mediated activation is predominantly regulated by synovial macrophages that have been shown to drive onset [40,41], elongation [42], and flare-up [43] during AIA. In a previous study we found that mice that express nonfunctional FcγRI and FcγRIII and developing comparable adaptive immunity with controls showed a significantly lower knee joint swelling. In this study, both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice showed however comparable swelling with controls. These results suggest that FcγRI and FcγRIII present on macrophages act in concert with respect to swelling. In the absence of one receptor its function may be fully compensated by the other FcγR. These receptors that are expressed on mononuclear cells [44] are involved in the respiratory burst on cross-linking by IgG-ICs. As a result these cells release vasoactive products such as oxygen and nitrogen radicals and histamine that are highly involved in vascular leakage leading to enhanced ^{99m}Tc uptake in the joint.

In humans, FcγRIIIA has been suggested to play a role in the pathogenesis of RA and a close correlation was found between FcγRIIIA expression and the location of both synovitis and extracellular features [45]. A recent study suggests a dominant role for FcγRIIIA in the induction of both TNF-α and IL-1α production by human macrophages in RA after receptor ligation by small ICs [46]. Furthermore many animal studies have shown that FcγRIII is crucial in IC-dependent diseases raised in skin [47], kidney [48], or lung [49]. A reverse passive arthus reaction is inhibited within FcγRIII$^{-/-}$ mice [23]. Recently, we also found that arthritis elicited by ICs only is also very strongly FcγRIII-dependent (Nabbe K, Van Lent P, Blom AB, Holthuysen A, Verbeek S, Van den Berg W, manuscript in preparation). The reason why FcγRIII does not play an important role within AIA may be that apart from ICs T cells also play an important role in chronicity within this model. During AIA, mBSA-specific Th1 cells accumulate in the arthritic joint thereby releasing interferon (IFN)-γ. IFN-γ is one of the most potent cytokines involved in up-regulation of FcγRI on macrophages [50]. Local IFN-γ production may cause a shift in FcγR expression from FcγRIII to FcγRI and may explain the redundancy found within this study.

The inhibiting FcγRII seems to be of utmost importance in both the acute and chronic phase of synovial inflammation and absence leads to significantly higher inflammation. FcγRII is expressed on various hematopoietic cells and is a negative regulator of FcγRIII receptor on B cells and macrophages [51] and probably also of FcγRI [52]. FcγRII may act as a negative regulator of antibody production and/or local IC-triggered activation. As most of the anti-mBSA isotype titers were elevated, this may be responsible for a higher IC load within the knee joint.

Although we did not find differences in the IC load in the knee joints of FcγRII$^{-/-}$ and controls at day 7 after AIA using immunolocalization, differences may have been present at earlier time points of this arthritis. Local IC-triggered activation in the joint may further be different. Absence of the FcγRII on macrophages has been shown to enhance the release of inflammatory mediators such as IL-1 and TNF-α [53] that are highly involved in synovial inflamma-

tion. Comparing two doses of mBSA we found that only the high dose (60 μg) of mBSA was FcγRII-dependent during the complete 7-day course of arthritis. Immune complexes drive inflammation by both activating Fc receptors and complement and the two systems seemed to be co-dominant in the early arthus reaction [54]. Our results suggest that after the arthus, in the developing phase of chronicity of experimental arthritis, higher IC doses may be more FcγR-dependent if compared with lower IC doses.

Previous studies have shown that macrophages are crucial in cartilage destruction seen during AIA [38-41]. Macrophages may function either indirectly by attracting inflammatory cells or directly as important producers of cytokines [55] and enzymes [56], and in RA activation of macrophages may be T cell-dependent [57,58]. During AIA, two phases of cartilage destruction are observed. The first early phase, which is characterized by proteoglycan loss, is mediated by aggrecanase, a member of the ADAMS (a desintegrin and metalloproteinase) family. The second phase that starts at approximately day 5 after AIA induction, is characterized by MMP-mediated matrix destruction resulting in irreversible cartilage erosion [32]. Because no difference was found in PG breakdown nor NITEGE staining in cartilage surfaces of arthritic knee joints of FcγR-, FcγRIII-, or FcγRII-deficient mice and their wild-type controls (data not shown), this suggests that these receptors are not important or redundant in aggrecanase-induced cartilage damage.

In contrast, MMP-mediated cartilage destruction as measured by VDIPEN staining was significantly lower in FcγRI$^{-/-}$ but not in FcγRIII$^{-/-}$ arthritic knee joints. The latter indicates that FcγRI rather than FcγRIII is involved in late severe cartilage destruction. This type of cartilage destruction only occurs in experimental models, containing an IC component [32]. Other arthritis models induced by bacterial or yeast cell walls never resulted in severe cartilage destruction and also expression of VDIPEN neoepitopes was fully absent [32]. As we showed earlier, the activatory FcγR are probably involved in activation of latent MMPs present in the cartilage matrix of arthritic knees, binding of FcγRI may lead to activation of synovial macrophages leading to selective production of pro-MMP-activating factors. Although erosion was still mild at day 7 after AIA induction, there was a tendency that erosion was lower in cartilage layers of arthritic knees of FcγRI$^{-/-}$ mice. Differences may diverge more at later time points when erosion becomes more severe within this model.

In contrast, in the absence of FcγRII significantly higher MMP-mediated destruction was found within the cartilage layers of the arthritic joints. First, this may be because of an elevated load of IC within the joint as a result of higher anti-mBSA titers and may explain the higher VDIPEN expression in the 60 μg mBSA group. Furthermore, macrophages deficient for FcγRII produce higher IL-1 concentrations on activation with ICs [39]. As interleukin-1 is the dominant cytokine involved in VDIPEN expression during AIA [59] this also may contribute to the higher VDIPEN expression in the cartilage. Moreover, the absence of FcγRII may elevate mechanisms involved in activation of pro-MMPs into their active

form. A higher concentration of enzymes released by macrophages and/or polymorphonuclear leukocytes such as elastase may be responsible for more efficient activation of pro-MMPs [60]. Whether FcγRII contributes to higher joint inflammation and severe cartilage destruction either by elevation of anti-mBSA antibodies and subsequent raise of ICs and/or by elevation of local hypersensitivity of macrophages to ICs is momentarily under investigation by blocking FcγRII at the onset of AIA using anti-FcγRII antibodies. The above results suggest that expression of the activatory FcγRI on the surface of local cells present in the knee joint during T cell-dependent AIA is the dominant activatory Fc receptor involved in cartilage destruction, whereas the inhibiting FcγRII$^{-/-}$ may be an important regulating receptor of severe cartilage destruction. During late phases of human arthritis, monocytes/macrophages are the dominant hematopoietic cells present in the RA joint and a strong correlation between the number of macrophages and erosion of the cartilage matrix was found during RA [61]. Disturbance in the balance between activating FcγRI and FcγRIII receptors and the inhibiting FcγRII receptor on synovial macrophages may have important implications for cartilage destruction during arthritis and the inhibiting FcγRII may form a new target for therapeutic intervention of cartilage destruction within this destructive disease.

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Chapter 3

The inhibitory receptor FcγRII reduces joint inflammation and destruction in experimental immune complex-mediated arthritides not only by inhibition of FcγRI/III but also by efficient clearance and endocytosis of immune complexes

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Studies in FcγRII$^{-/-}$ mice identified the inhibitory function of this receptor in joint inflammation and cartilage destruction induced with immune complexes (ICs). To extend our insight in the role of FcγRII in arthritis, we explored the role of FcγRII in the absence of activating receptors I and III using FcγRI/III$^{-/-}$ as well as FcγRI/II/III$^{-/-}$ mice. When antigen-induced arthritis (AIA) was elicited , which is a mixture of T cell and IC-driven inflammation, arthritis was almost absent at day 7 in FcγRI/III$^{-/-}$ mice. Remarkably, in FcγRI/II/III$^{-/-}$ mice, this model induced a tremendously increased arthritis as compared to wild-type controls. This implies that FcγRII regulates joint inflammation also in the abscence of activating FcγRI and III. To confirm the IC specificity of this finding, similar studies were done with ICs or zymosan as arthritogenic stimuli. Strongly elevated inflammation was found in FcγRI/II/III$^{-/-}$ mice with IC but not with zymosan. Clearance studies identified accumulation of IgG in the knee joint in the absence of FcγRII. Moreover, macrophages expressing only FcγRII showed prominent endocytosis of preformed soluble ICs not different from controls. In total absence of FcγR (FcγRI/II/III$^{-/-}$), macrophages completely failed to endocytose ICs. Although joint inflammation was much higher in AIA arthritic knee joints of FcγRI/II/III$^{-/-}$ and the inflammatory cells still expressed an inflammatory phenotype, severe cartilage destruction (MMP-mediated neoepitopes in the matrix and chondrocyte death) was completely prevented in contrast to the marked destruction which was observed in the wild-type. Our study indicates that FcγRII reduces joint inflammation in the absence of activating FcγR by promoting endocytosis and clearance of ICs from the joint. Infiltrating cells, which fail to express activating FcγR although they still become stimulated are no longer capable of inducing severe cartilage destruction.

Rheumatoid arthritis (RA) is a heterogenous chronic joint disease characterised by invasion of leucocytes and local synovium activation, which leads to severe destruction of cartilage and bone [1]. The most prominent leucocyte present within the inflamed synovium is the macrophage. A strong correlation was found between the number of activated macrophages and severe cartilage destruction [2]. In a normal joint, macrophages are comprised within the intima layer which covers the surface of the synovium [3]. In RA, synovial macrophages become activated, resulting in the release of chemokines, cytokines, and enzymes involved in regulation of joint inflammation and cartilage/bone destruction [4, 5].

The mechanism by which synovial intima macrophages become activated during RA is not known. One of the potential candidates are IgG-containing immune complexes (ICs). They are abundantly found in RA synovial fluid, synovium, and surface layers of the cartilage [6]. In previous studies we have found that lining macrophages are of utmost importance in both onset and prolongation of experimental murine arthritis. When synovial intima macrophages were selectively depleted from the knee joint either before induction or during immune complex

(IC)-mediated arthritides like collagen type II (CIA) or antigen-induced arthritis (AIA), onset and course of arthritis was largely reduced [7-9].

IgG-containing ICs communicate with lining macrophages using Fcγ receptors (FcγRs) [10]. In the mouse, three classes of FcγR have been described. FcγRI and III are activating receptors and lead to elevation of intracellular signaling after binding of ICs [11-12]. The third class is FcγRII, which can co-ligate with either FcγRI or FcγRIII, resulting in inhibition of intracellular signaling [13]. Coordinate expression of activating and inhibiting FcγR on synovial lining cells has been shown to regulate both joint inflammation and severe cartilage destruction [10]. The inhibiting FcγRII exists as two isoforms, FcγRIIb1 and FcγRIIb2, differing by a 47-amino acid insertion in the intracytoplasmatic domain of FcγRII encoded by the first exon of the FcγRII gene [14]. The in vivo role of FcγRII was extensively studied using FcγRII-deficient mice and it is generally agreed that inhibition occurs only when FcγRII is co-clustered with ITAM-bearing receptors [15,16]. The inhibitory function is mediated by the inositol phosphatase SHIP which associates with the phosphorylated ITIM of FcγRII via the SHIP SH2 domain [17]. However, in vitro studies also suggested other biological functions for FcγRII. By transferring cDNA of both FcγRII isoforms into fibroblastic cell lines which do not express FcγR, it was found that FcγRIIb2 is involved in endocytosis and enhancement of antigen presentation [18-20]. FcγRIIb1, which is preferentially expressed in B lymphocytes, lacks immune internalization properties, yet it inhibits B-cell activation and subsequent antibody production when cross-linked to membrane Ig. This suggests that FcγRII, apart from inhibiting activating FcγR, may also have other important functions in vivo.

In the present study, we investigated the in vivo role of FcγRII, uncoupled from its function as inhibitor of activating FcγR, in regulating joint inflammation and severe cartilage destruction in models of IC-mediated arthritis using mice which were made deficient for either both activating FcγR (FcγRI/III$^{-/-}$) or all three FcγR (FcγRI/II/III$^{-/-}$). We found that FcγRII is a major regulator of joint inflammation by promoting clearance of ICs by synovial lining cells. Furthermore activating FcγR on inflammatory cells appeared to be prerequisites for severe irreversible cartilage destruction.

Material and Methods

Animals

FcγRI and FcγRIII$^{-/-}$ were made deficient for the ligand-binding α-chain of FcγRI [21] and FcγRIII [22], respectively. FcγRIII$^{-/-}$ were back-crossed to the C57BL/6 background for 12 generations. FcγRIIb$^{-/-}$ were developed by Dr.Takai [15] in the 129Ola (H-2b) and C57BL6 (H-2b) background. FcγRI$^{-/-}$ was made in the 129Ola/C57Bl/6 background. FcγRII/III$^{-/-}$ and their controls (control 1) were developed in the 129Ola/C57Bl/6 background. Intercrossing led to FcγRI/II/III$^{-/-}$ in the 129Ola/C57Bl/6/balb/c (enriched for C57Bl/6), as were their triple controls (control 2). Control C57Bl/6 and 129Ola/C57Bl/6 were derived from Jackson laboratories (Bar

Harbor, ME) and bred in our own facilities. Homozygous mutants and their wild-type (WT) controls, aged 10 to 12 weeks, were used in the experiments.

Humoral immunity against mBSA

Antibodies of various isotypes (IgG, IgG1, IgG2a, IgG2b, IgG3) directed against methylated bovine serum albumin (mBSA) were measured in sera of individual mice with an enzyme-linked immunosorbent assay (ELISA). Antigen was coated on microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) at a concentration of 100 μg/ml. Antibody titers were assessed by two-fold serial dilution of the sera followed by detection of bound mouse Ig with 1:500 diluted peroxidase-conjugated rabbit anti-mouse Ig (Miles Laboratories Inc., Elkhart, IN). O-Phenylenediamine (1 mg/ml; Sigma, St. Louis, MO) was used as substrate for peroxidase, and the antibody titer was determined by using 50% of the maximal extinction as an end-point.

Cellular immunity against mBSA

Mouse spleen cells were isolated and washed in RPMI supplemented with 10% fetal calf serum, glutamin (2 mM) and pyruvate (1 mM). Erythrocytes were lysed by treatment of the cells with an 0.16 M NH_4CL solution in 0.17 M Tris, pH 7.2, for 5 minutes. After two washes in RPMI, the cells were plated on plastic T flasks (75 mm^2) from Falcon Plastics (Oxnard, CA). After 60 minutes of incubation at 37°C, the nonadherent cells, were harvested by aspiration and two 4- to 5-ml RPMI washes of the adherent cells. Hundred microliter of RPMI containing 1 x 10^5 T-cell-enriched spleen cells were placed in each well of a sterile, U-bottomed polystyrene microculture plate (Costar, Cambridge, MA). Antigens or mitogens were added in another 100 μl to give a total volume of 200 μl, and final concentrations of antigen of 50, 25, 12, 6, and 3 μg/ml. Cultures were maintained at 37 °C in a humified atmosphere of 2% CO_2 for 4 days. Sixteen hours before harvesting, 1 μCi of [^{3}H]-thymidine (6.7 μCi/mmole from New England Nuclear, Boston, MA) was added in 25 μl of RPMI. Cultures were harvested with a cell harvester and [^{3}H]-thymidine incorporation was quantified using a Micro Beta-plate reader (Perkin, The Netherlands).

Induction of experimental arthritis

AIA was induced by injecting 60 μg of mBSA in 6 μl PBS directly into the knee joints of mice that were previously immunized with that antigen. Mice were immunized with 100 μg of mBSA (Sigma, Zwijndrecht, The Netherlands), emulsified in 100 μl Freunds complete adjuvant (CFA). Injections were divided over both flanks and footpath of the forelegs. Heat-killed Bordetella pertussis was administered intraperitoneally as an additional adjuvant. Two subcutaneous booster injections with 50 μg mBSA/CFA were given in the neck region one week after the initial immunization. Two weeks after these injections, arthritis was induced into the right knee joint, resulting in chronic arthritis.

Immune complex arthritis (ICA) was passively induced in knee joints of mice [23]. Three microgram of lysozyme in 6 μl were injected directly into the knee

joint of mice that previously were given anti-lysosyme antibodies intravenously. An acute arthritis develops, which became maximal at day 3 and waned thereafter.

A non-IC-mediated zymosan-induced arthritis (ZIA) was induced by injecting 180 μg sterilized zymosan in 6 μl PBS into the knee joint.

^{99m}Tc Uptake measurements

Joint inflammation was measured by ^{99m}Tc pertechnetate uptake in the knee joint. This method was shown earlier to correlate well with histological findings [24]. Briefly, mice were injected intraperitoneally with 12 μCi ^{99m}Tc and subsequently sedated with chloralhydrate. Thirty minutes thereafter, gamma radiation was assessed by use of a collimated Na-I-scintillation crystal with the knee in a fixed position. Arthritis was scored as the ratio of the ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R:L ratios > 1.1 were taken to indicate inflammation of the right knee joint.

Histology

Total knee joints were dissected, fixed in phosphate buffered formalin (pH 7.4), decalcified in 5% buffered formic acid, and subsequently embedded in paraffin wax. Semiserial frontal whole knee joint sections (7 μm) were stained with hematoxylin and eosin (H&E) or safranin O and fast green. The severity of joint inflammation was determined using an arbitrary score (0 to 3). Infiltrate and exudate were scored separately. Scoring was performed in a blinded manner by two independent observers: 0, no cells; 1, mild cellularity; 2, moderate cellularity; 3, maximal cellularity.

Endocytosis and clearance of IgG immune complexes

Endocytosis and clearance of IgG ICs were studied both in vivo and in vitro. The clearance of IgG-containing ICs from arthritic knee joints of FcγRI/II/III$^{-/-}$ and FcγRI/III$^{-/-}$ mice was studied using anti-IgG immunolocalization. In one group, AIA was induced whereas in a second group knee joints were injected with 6 μg of heat-aggregated IgG. The latter was made by heating rabbit-IgG during 30 minutes at 61°C. Knee joints were isolated 7 days after AIA induction or 8 hours after injection of aggregated IgG. Paraffin-embedded total knee joint sections were pretreated with hyaluronidase ABC and additionally stained with either goat anti-mouse peroxidase or goat anti-rabbit peroxidase overnight. Induction of the peroxidase product was detected using diaminobenzidine (0.5 mg/ml). Sections were counterstained with H&E.

Isolation of peritoneal macrophages from mice previously injected with thioglycolate

ICs were preformed by incubating soluble fluorescein isothiocyanate (FITC)-labeled OVA (Molecular Probes, Leiden, The Netherlands) with 25 μg/ml polyclonal OVA-specific rabbit IgG (rIgG OVA; Sigma-Aldrich, Zwijndrecht, The Netherlands) for 30 minutes at 37°C in propylene tubes. Fifty thousand peritoneal macrophages were added to FACS tubes containing OVA-ICs or soluble OVA and incubated for 15 minutes at 37°C. Cells were washed twice and resuspended in presence of 0.4% (w/v) trypan blue (Sigma-Aldrich), which quenches extracellular, but not intracellular, fluorescence. Flow cytometry

was performed with FACScan. The mean fluorescence value of six measurements is shown.

Immunohistochemical staining of myeloid related proteins (MRP)8 and 14

Rabbit anti-sera against recombinant murine MRP8 (α-MRP8) and MRP14 (α-MRP14) were produced as described earlier [25]. Monospecificity of antibodies was analyzed by immunoreactivity against recombinant MRP8 and MRP14 and Western blot analysis of lysates of granulocytes [25]. Formalin-fixed sections of knee joints were stained using a final antibody concentration of 1 μg/ml. Primary antibodies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany). MRP8- and MRP14-positive cells present in the joint cavity and synovial lining were determined and expressed as percentage of the total cell population, using an arbitrary score (0:0%, 1:1 to 30%, 2:31 to 70%, 3:71 to 100%).

Immunolocalization of MMP-induced neoepitope (VDIPEN)

For immunohistochemical analysis, sections were deparaffinized, rehydrated and digested with chondroitinase ABC (Sigma, 0.25 U/ml, 0.1 M Tris-HCL, pH 8.0) for 1 hour at 37°C, to remove chondroitine sulphate from the proteoglycans. Sections were then treated with 1% H_2O_2 in methanol for 20 minutes and subsequently for 5 minutes with 0.1% (v/v) triton X-100 in PBS. After incubation with 1.5% (v/v) normal goat serum for 20 minutes, sections were incubated with affinity-purified anti-VDIPEN IgG overnight at 4°C. These antibodies were kindly provided by Irwin Singer and Ellen Bayne (Merck Research Laboratories,Rahway,NJ) and have been extensively characterized before [26,27]. In addition, sections were incubated with biotinylated goat anti-rabbit IgG and binding was detected using avidin-peroxidase staining (Elite kit, Vector Laboratories, Inc., Burlingham, CA). Induction of the peroxidase product was detected using nickel enhancement and counterstaining was performed with orange G (2%) for 5 minutes.

Measurement and characterization of chondrocyte death

Chondrocyte death was determined at day 7 after AIA induction in total knee joint sections stained with H&E. Chondrocyte death was determined as percentage of the area of the cartilage containing empty lacunae in relation to the total area.

Results

Role of the inhibitory FcγRII in the absence of activating FcγR during antigen-induced arthritis

To investigate the role of FcγRII in the absence of activating FcγR, we induced AIA in knee joints of FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ mice. As the absence of FcγR may alter the immunological response against methylated BSA during immunization of these mice, thereby impairing the onset and course of arthri-

Table 1 Cellular and humoral immunity four weeks after immunization with mBSA/CFA

	Cellular Immunity (SI)					Humoral Immunity (titer)				
	50	25	12	6	3	IgG	IgG1	IgG2a	IgG2b	IgG3
WT control	3(1)	5(1)	6(2)	7(1)	5(1)	13(2)	11(1)	12(2)	11(1)	10(1)
FcγRI/III$^{-/-}$	5(2)	7(1)	8(1)	8(0)	5(0)	14(2)	13(1)	13(1)	11(1)	11(0)
WT control	1(1)	2(1)	5(0)	6(1)	4(1)	17(1)	14(2)	13(1)	14(0)	10(1)
FcγRI/II/III$^{-/-}$	1(0)	4(2)	6(1)	7(1)	5(0)	18(1)	16(2)	15(1)	14(1)	11(2)

Cellular immunity was measured by spleen lymphocyte proliferation and expressed as stimulation index (ratio of T cells stimulated with various concentrations of mBSA (50, 25, 12, 6, 3 μg/ml) and T cells not stimulated). Values represent the mean ± SD of three groups of 2 mice each. Humoral immunity was measured as antibody production against mBSA and various isotypes were determined using ELISA. Note that IgG1, IgG2a and IgG3 levels were elevated (2-4 times), whereas IgG2b was not elevated in both FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ when compared to controls.

tis, we first tested cellular and humoral immunity to mBSA, 3 weeks after immunization. Cellular immunity as measured by spleen lymphocyte stimulation (LST) against various concentrations of mBSA showed no significant differences between the knockout (KO) and their controls (Table 1). In addition, humoral immunity was measured by ELISA. Total IgG, IgG1, IgG2a, and IgG3 anti-mBSA levels were two to four times higher, whereas IgG2b levels were not different in sera of both immunized FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ when compared to their WT controls (Table 1).

Subsequently, AIA was induced and knee joint swelling was determined at various time-points after induction. We found that inflammation in arthritic knee joints of FcγRI/III$^{-/-}$ was significantly lower both at day 3 and day 7 when compared to arthritic controls (Figure 1A). Interestingly, inflammation in arthritic knee joints of FcγRI/II/III$^{-/-}$ was not different from that seen in control knee joints both at day 3 and 7 after AIA induction (Figure 1B).

To further verify these observations, histology of total arthritic knee joints was investigated. At day 7 after AIA induction, FcγRI/III$^{-/-}$ mice showed that although IgG2a antibody titers were much higher, exudate and infiltrate was significantly lower (90% and 87%, respectively) when compared to WT controls (Figure 2, A and D, versus WT control, Figure 2E). In contrast, at day 7 after AIA induction in knees

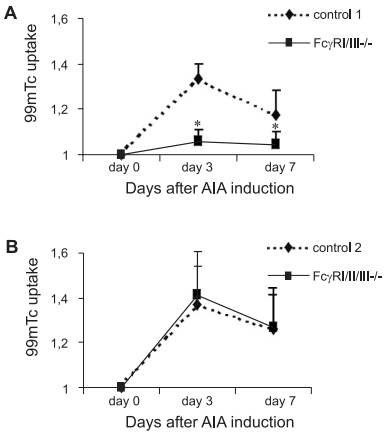

Figure 1
Inflammation of the knee joint, expressing cellular activation, was determined as R:L ratios of ^{99m}Tc uptake at various days (3 and 7) after injection of 60μg mBSA in the right knee joints of mBSA-immunized FcγRI/III$^{-/-}$ (A) and FcγRI/II/III$^{-/-}$ mice(B). In the left knee joints PBS was injected. Values represent the mean ± SD of 7 mice. Data were evaluated using the Wilcoxon rank test (*, $P < 0.05$). Note that swelling was almost absent in knee joints of arthritic FcγRI/III$^{-/-}$, whereas similar joint inflammation was found in arthritic FcγRI/II/III$^{-/-}$.

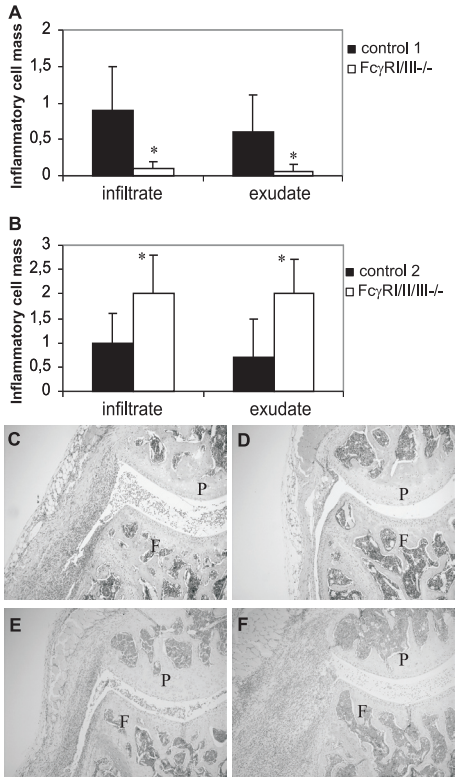

Figure 2
Frontal sections of knee joints 7 days after induction of AIA in FcγRI/III$^{-/-}$ (A), FcγRI/II/III$^{-/-}$ (B) and their wild-type controls 1 and 2. The amount of cells present in the synovium (infiltrate) and in the knee joint cavity (exudate) was determined using an arbitrary scale from 0 to 3: 0, no cells; 1, minor; 2, moderate; 3, maximal. The amount of cells was determined independently by two blinded observers. Data are the mean of 7 mice. Significance was tested using the Wilcoxon rank test (*, P<0.05). Original magnification of the photographs is 100X. F, femur; P, patella. Note the significantly lower inflammatory cell mass in knee joints of FcγRI/III$^{-/-}$ (A and D versus WT control C) and the significantly higher cell mass in FcγRI/II/III$^{-/-}$ knee joints (B and F versus WT control E) when compared to WT controls.

of FcγRI/II/III$^{-/-}$, joint inflammation appeared to be markedly higher when compared to their controls (exudate and infiltrate were respectively 200% and 120% higher (Figure 2, B and F versus WT control, Figure 2E)), suggesting that FcγRII is an important regulator of joint inflammation in the absence of activating FcγR.

FcγRII regulation of joint inflammation is specific for immune complexes

To further investigate whether FcγRII regulation of joint inflammation in the absence of activating FcγR is specific for ICs (and not, for example, by T cells also involved in AIA), we induced arthritis solely by ICs. ICA was passively induced by injecting lysozyme in knee joints of mice that were previously given anti-lysozyme antibodies. Histology taken at day 3 after arthritis induction showed that joint inflammation was almost completely prevented in FcγRI/III$^{-/-}$, whereas substantial arthritis was found in their WT controls (Figure 3A). When ICA was induced in knee joints of FcγRI/II/III$^{-/-}$, the inflammatory cell mass as measured at day 1 and 3 was in line with that found in AIA, again significantly higher when compared to WT controls. At day 1, exudates and infiltrate were 310% and 60%, respectively (Figure 3B), and at day 3, 2200% and 270% higher (Figure 3C).

To further substantiate the specificity for ICs, we additionally injected zymosan in the knee joints of FcγRI/II/III$^{-/-}$. The inflammatory cell mass measured at day 3 after ZIA induction was not different from controls, suggesting that knee joints of these mice develop a normal inflammatory response after injection with zymosan (Figure 3D).

FcγRII is involved in efficient endocytosis and clearance of ICs

One of the reasons why joint inflammation is elevated in FcγRI/II/III$^{-/-}$ mice may be an impaired endocytosis and clearance of ICs from the joint.

To investigate whether FcγRII is in-

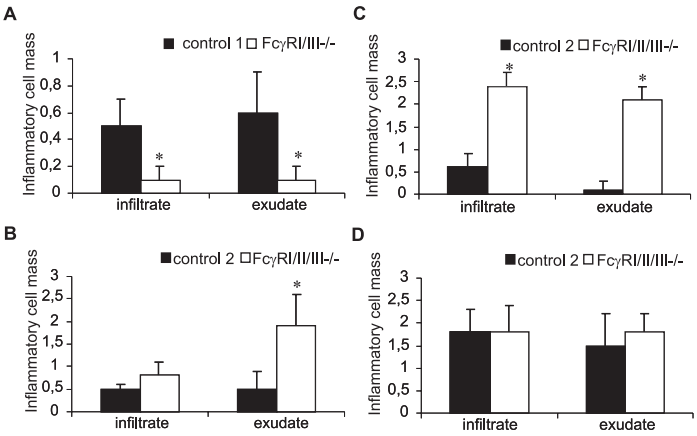

Figure 3
Inflammatory cell mass in knee joints, 3 days after induction of ICA in FcγRI/III$^{-/-}$ (A), 1 and 3 days after ICA in FcγRI/II/III$^{-/-}$ (B and C) or 3 days after induction of zymosan-induced arthritis (D) in FcγRI/II/III$^{-/-}$ and their WT controls 1 and 2. Data are the mean of 7 mice. Significance was tested using the Wilcoxon rank test (*, $P<0.05$). Original magnification of the photographs is X 100.F, femur; P, patella. Note the significantly lower inflammatory cell mass after ICA induction in knee joints of FcγRI/III$^{-/-}$ (A) and the significantly higher cell mass at day 1 and 3 in FcγRI/II/III$^{-/-}$ knee joints (B and C) and the comparable cell mass at day 3 after ZIA (D) when compared to WT controls.

volved in endocytosis of IgG containing ICs, we first investigated the presence of murine IgG, localized within the arthritic joints at day 7 after AIA induction using immunolocalization. No significant differences in IgG deposition was found in the knee joints of FcγRI/III$^{-/-}$ mice and their WT controls, suggesting an effective clearance of IgG containing ICs in the presence of only FcγRII (Figure 4B versus WT control, Figure 4A). In contrast, arthritic knee joints of FcγRI/II/III$^{-/-}$ contained large amounts of IgG, suggesting that removal of ICs is retarded (Figure 4D versus WT controls, Figure 4C). To confirm this finding, heat-aggregated IgG was injected in the knee joints of FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ mice and their WT controls. Immunolocalization of IgG showed that 8 hours after injection significantly more IgG was detected in FcγRI/II/III$^{-/-}$ knee joints which was mainly bound to the synovial lining layer (Figure 5D versus WT control, Figure 5E). Similar intensity of staining was found when aggregated IgG was injected in knee joints of FcγRI/III$^{-/-}$ and their controls (Figure 5B versus WT control, Figure 5A).

To further analyze FcγRII function on macrophages, thioglycollate-induced peritoneal macrophages were isolated. When macrophages expressing only FcγRII (FcγRI/III$^{-/-}$) were pre-incubated with pre-formed FITC-labeled OVA-IgG ICs, prominent endocytosis was found

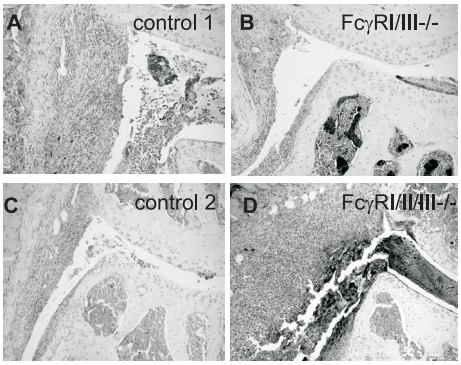

Figure 4
Presence of IgG-containing ICs, detected by immunolocalization, in knee joints of various KO mice, 7 days after induction of antigen-induced arthritis. Note that IgG is present in large amounts in arthritic FcγRI/II/III$^{-/-}$ knee joints when compared to their WT controls (D versus WT control C). No difference in amounts of IgG was found in arthritic knee joints of FcγRI/III$^{-/-}$ when compared to their controls (B versus WT control A). Original magnification, X 100.

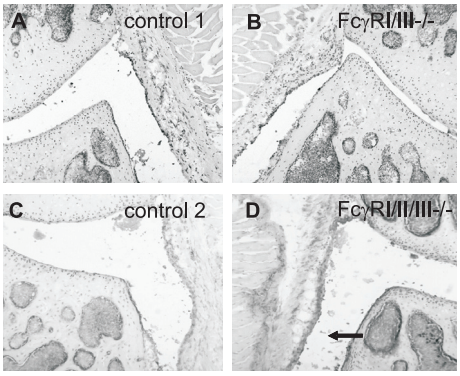

Figure 5
Presence of heat-aggregated IgG detected by immunolocalization, 8 hours after injection in knee joints of various KO mice. Heat-aggregated rabbit IgG is less efficiently cleared (arrow) when injected in FcγRI/II/III$^{-/-}$ knee joints in comparison to WT controls 2 (D versus WT control C). No differences are found between knee joints of FcγRI/III$^{-/-}$ and their WT controls (B versus WT control A). Original magnification, X 100.

not different from control macrophages (Figure 6B versus control, Figure 6A). Interestingly when FcγRII was also absent (FcγRI/II/III$^{-/-}$), endocytosis of ICs was completely prevented (Figure 6C versus control, Figure 6A).

Type and activation state of inflammatory cells in arthritic knee joints of FcγRI/II/III$^{-/-}$ mice

To further analyze the composition of the inflammatory cell mass within the arthritic FcγRI/II/III$^{-/-}$ knee joint, we next investigated the type and activation state of the inflammatory cells using immunolocalization. PMN and monocyte/macrophage ratios were determined by immunolocalization using NIMP-R14, which stains PMN specifically. At day 7 after AIA induction in FcγRI/II/III$^{-/-}$ knee joints, the majority of inflammatory cells appeared to be monocytes (ratio monocytes/PMN 60-40) and no differences were found between KO and their controls. In addition, we determined the pro-inflammatory phenotype of the infiltrated cells. In FcγRI/II/III$^{-/-}$ mice, the infiltrated cells in the arthritic joint displayed an activated phenotype, according to high expression of MRP8 and 14 (respectively 47% and 92%: Figure 7, A and C versus WT control, Figure 7B), whereas infiltrated cells in the joints of FcγRI/III$^{-/-}$ mice failed to express these activation markers (data not shown). In fact this implies that FcγRII can prevent cellular activation even in the absence of FcγRI/III.

Activated inflammatory cells in the absence of activation FcγR fail to induce severe cartilage destruction

As the majority of the infiltrated cells in the FcγRI/II/III$^{-/-}$ knee joints were activated, we additionally investigated

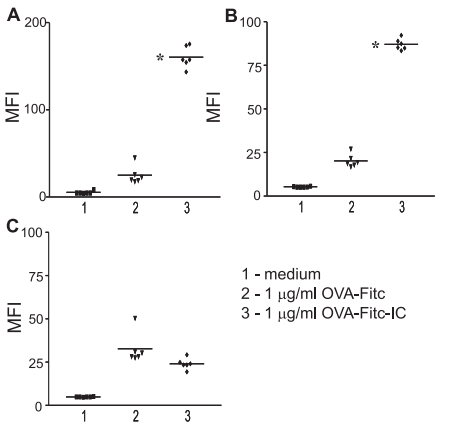

Figure 6
Endocytosis of soluble fluorescent ovalbumin–IgG ICs by peritoneal macrophages derived from FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$. Note that ICs are efficiently endocytosed by macrophages expressing only FcγRII (FcγRI/III$^{-/-}$) not different from controls (B versus WT control A). When macrophages missing all FcγR (FcγRI/II/III$^{-/-}$) were used, endocytosis of ICs was blocked (C versus WT control A). Peritoneal macrophages of WT littermates of FcγRI/III$^{-/-}$ and FcγRI/II/III$^{-/-}$ were used as controls. As uptake of IC by both control cells was similar, only control cells of FcγRI/II/III$^{-/-}$ are shown. MFI, mean fluorescent intensity. Data are the mean of three different experiments. Significance was tested using the Wilcoxon rank test (*, $P<0.05$).

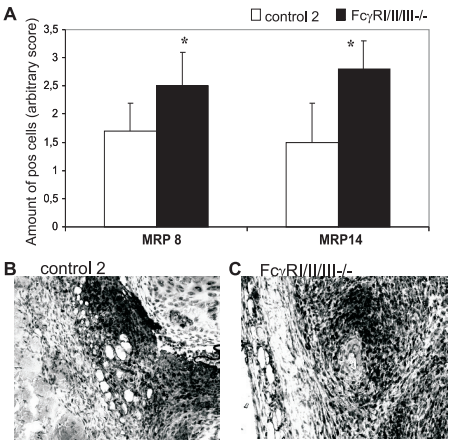

Figure 7
Expression of activation markers MRP8 and 14 in synovial lining and joint cavity in FcγRI/II/III$^{-/-}$ and their WT controls at day 7 after AIA induction. Note the significantly higher expression of both MRP8 (A and C versus WT control B) and MRP14 (A). Data are the mean of 7 mice. Significance was tested using the Wilcoxon rank test (*, P<0.05). Original magnification, X 400.

whether these cells were capable of inducing severe cartilage destruction like metalloproteinase (MMP)-induced damage and chondrocyte death. MMPs are crucial in degradation of aggrecan and collagen, leading to irreversible cartilage destruction. MMPs degrade aggrecan leaving the C-terminal ending with the amino acid sequence VDIPEN which can be detected by specific antibodies around day 5 after induction of AIA [27]. For this reason, AIA day 7 was taken to detect VDIPEN expression in the cartilage matrix.

The amount of VDIPEN was measured by determining the percentage of the area of cartilage expressing VDIPEN. In most investigated knee joints of WT mice injected with 60 μg of mBSA, VDIPEN staining was found particularly in the cartilage layers of tibia and femur (Figure 8, A, C, and E). In arthritic knees of FcγRI/III$^{-/-}$, VDIPEN expression was completely absent when compared to WT controls (Figure 8, A and D, versus WT control, Figure 8C). Interestingly, in FcγRI/II/III$^{-/-}$ arthritic joints, although much more joint inflammation was found which abundantly expressed the activation markers MRP8 and 14, VDIPEN was virtually absent when compared to arthritic controls (Figure 8, B and F, versus WT control, Figure 8E). This confirms again that only activating FcγR mediate severe cartilage destruction and that this prerequisite also holds in condition of abundant inflammatory cell influx showing an activated phenotype.

In addition, chondrocyte death was measured by determining the percentage of the area of cartilage with empty lacunae. Chondrocyte death varied between 5% to 40% in various cartilage layers of the WT arthritic knee joints (Figure 9, A, C, and E). Chondrocyte death was absent in arthritic FcγRI/III$^{-/-}$ knee joints (Figure 9, A and D, versus WT control, Figure 9C). Again in arthritic FcγRI/II/III$^{-/-}$, despite its high joint inflammation, chondrocyte death was completely absent (Figure 9, B and F, versus WT control, Figure 9E).

Discussion

The in vivo role of FcγRII has been extensively studied using FcγRII-deficient mice. Induction of IC-mediated inflammation within these mice caused a significantly elevated inflammation when compared to controls. In general, the function of FcγRII as a major inhibitor of the activatory FcγR is highlighted. In the present study, we demonstrate that in the absence of activating FcγR, the inhibiting FcγRII still functions as an important

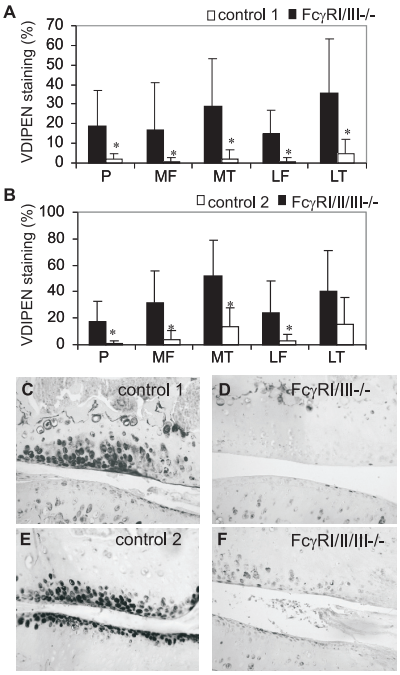

Figure 8
Expression of VDIPEN neoepitopes in knee joints of FcγRI/III$^{-/-}$ (A), FcγRI/II/III$^{-/-}$ (B) and their WT controls 1 and 2, 7 days after AIA induction. VDIPEN staining was determined in various cartilage layers of the knee joint (P, patella; MF, medial femur; MT, medial tibia; LF, lateral femur; LT, lateral tibia). VDIPEN was expressed as percent positive staining of the total cartilage area. VDIPEN staining was almost absent in arthritic knee joints of both FcγRI/III$^{-/-}$ (A and D versus WT control C) as in FcγRI/II/III$^{-/-}$ (B and F versus WT control E). Data represent the mean ± SD of 7 mice and were statistically evaluated using the Wilcoxon rank test. * P<0.05.

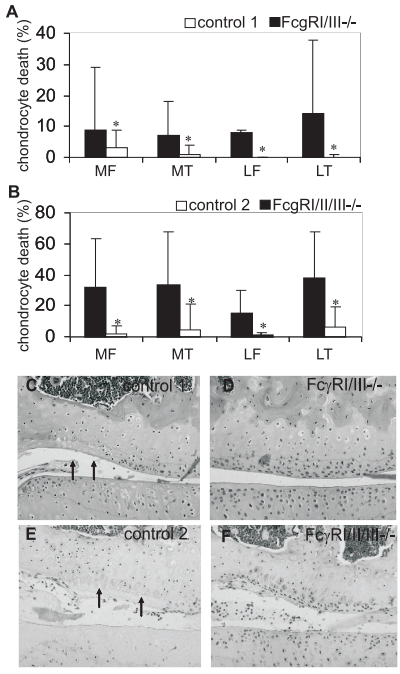

Figure 9
Measurement of chondrocyte death in cartilage layers of knee joints of FcγRI/III$^{-/-}$ (A), FcγRI/II/III$^{-/-}$ (B) and their WT controls 1 and 2, 7 days after AIA induction. Chondrocyte death was determined in various cartilage layers of the knee joint (MF, medial femur; MT, medial tibia; LF, lateral femur; LT, lateral tibia). Chondrocyte was expressed as percent empty lacunae (arrows in C and E) of the total cartilage area. Note that chondrocyte death was significantly lower in both FcγRI/III$^{-/-}$ (A and D versus WT control C) as in FcγRI/II/III$^{-/-}$ (B and F versus WT control E). Data represent the mean ± SD of 7 mice and were statistically evaluated using the Mann-Whitney U-test. * P<0.05.

down-regulator of synovial inflammation, which might be related to IC clearance and complement activation.

FcγRII, in the abscence of activating FcγR significantly reduced joint inflammation during T cell-mediated AIA. One of the explanations may be an altered T cell response. FcγR are expressed on precursors of T cells [28] and the absence of these receptors may have had an impact on the development of T cell reactivity and may explain the markedly elevated anti-mBSA IgG2a antibody responses in both immunized FcγRI/III$^{-/-}$ as FcγRI/II/III$^{-/-}$. However anti-mBSA T cell responses were found not to be significantly different and this may indicate that FcγRII present on resident synovial lining macrophages may be more important in regulating joint inflammation.

To further substantiate the involvement of FcγRII on synovial macrophages, arthritis was induced by local deposition of IC within the joint. In that model, arthritis is regulated by lining macrophages and not by T cells [23]. Comparable to

that seen in AIA, FcγRII, in the absence of activating FcγR, again strongly reduced joint inflammation. The FcγRII dependency appeared to be IC-specific since injection of zymosan directly into the knee joint of FcγRI/II/III$^{-/-}$ caused similar joint inflammation than when injected in WT and thus indicates that the joints of these mice develop a normal inflammatory response on non-IC triggers.

The most plausible function of FcγRII in joint inflammation in the abscence of activating FcγR is its role in clearance of IgG-ICs from the joint. Clearance of ICs is largely regulated by synovial lining cells and its efficiency is highly correlated to development of arthritis [29]. IgG-containing ICs activate complement. In the mouse IgG2a and IgG3 mediate complement via the classical pathway [30], whereas IgG1, when attached to cartilage layers uses the alternative pathway [31]. Co-dominance between complement and FcγR has previously been described [32]. Efficient removal of these ICs from the joint may lower the amount and course of complement activation within the joint, thereby lowering onset and/or prolongation of arthritis. This is in line with studies that show that complement is especially important in the onset, whereas at later time-points inflammation is more FcγR-mediated [33].

Within the joint, macrophages are crucial in clearance and endocytosis of ICs. In earlier in vitro studies using cDNA transfection, it was found that FcγRII mediates internalization and lysosomal degradation of IgG-antigen complexes [34,35]. In line with that, we now find that peritoneal macrophages from KO mice, which only express FcγRII and no activating FcγR, are still able to endocytose soluble ovalbumin-IgG complexes not different from controls, whereas in the absence of all FcγR, endocytosis is almost completely blocked. Moreover, when ICs were injected directly into the joint, clearance and endocytosis by lining cells were strongly retarded in the FcγRI/II/III$^{-/-}$. This suggests that in vivo FcγRII is a major receptor for endocytosis. In vivo studies using FcγRII-deficient mice have shown that FcγRII inhibited phagocytosis and clearance, and this was explained by inhibiting activating FcγR [36]. We now clearly demonstrate for the first time that in vivo, FcγRII also reduces inflammation by accelerating IC clearance and endocytosis. In contrast to our study, Mathis et al [31] found no involvement of FcγRII in the K/BXN serum transfer arthritis model. One explanation may be that arthritis within this model is regulated by anti-GPI antibodies of only the IgG1 isotype. These antibodies preferentially bind to FcγRIII which may largely be responsible for IC-removal within this model.

Removal of ICs from the joint is a combined action of leakage through the pores of the lining layer into the draining lymph vessels and lymph nodes, and binding and endocytosis by synovial lining cells. Synovial intima macrophages first meet these ICs and have been shown to be crucial in both onset as well as propagation of synovial inflammation [8,37]. This in contrast to synovial intima fibroblasts which fail to express FcγR. Activation of lining cells forms one of the crucial events in arthritis development. Transfer of early activated lining cells appeared to be sufficient to induce arthritis in normal rats [38].

It is generally accepted that FcγRII acts by coligating with activatory FcγRIII and probably also with FcγRI eventually leading to inactivation of synovial macrophages and reduced production of cytokines and chemokines [16]. In the present study we find that in the absence of all FcγR, IgG-ICs when injected into the joint still bind to intimal synovial cells. Moreover these cells express abundant MRP8/14 indicating that they still become activated and likely produce sufficient pro-inflammatory factors leading to pronounced joint inflammation. As FcγR are absent, ICs may bind to other receptors. A good candidate may be the promiscuous complement receptor 3 (CR3) [39]. The complement splitting product C3bi tightly binds to various antibody isotypes involved in IC formation [40]. C3bi may form the link between ICs and binding to the CR3 receptor on macropages not expressing FcγR and may mediate intracellular signaling leading to activation of the intimal macrophage.

The amount of inflammatory cell mass within an inflamed joint is often related to severe cartilage destruction. In the present study, we found a remarkable uncoupling between joint inflammation and severe cartilage destruction like MMP-mediated damage and chondrocyte death. Severe cartilage destruction seen during IC-mediated arthritides is mediated by metalloproteinases, which are released by chondrocytes in a latent pro-form within the cartilage matrix. Interleukin-1 appeared to be the master cytokine regulating MMP production by the chondrocyte [41]. Large amounts of inactive MMPs accumulate within the cartilage matrix and on activation lead to destruction of the collagen type II network and the proteoglycans embedded within this matrix [42]. The factors needed for this activation step are still unknown.

As inflammatory cells are capable of mediating activation of latent MMPs inside the cartilage matrix [43], the way in which these cells become activated within the joint seems crucial and recent studies by our lab suggest that activating FcγR are of utmost importance [10,21,44].

Binding of IC to activating FcγR (especially FcγRI) on macrophages may lead either to a higher production of MMP-activating factors or promote generation of mediators which inhibit these factors. MMP-activating factors may be other MMP [45], enzymes like plasmin [46], EMPRINN [47], or oxygen radicals [48], which all have been shown to possess the capacity of activating latent MMPs. Oxygen radicals have been shown to be abundantly released by macrophages after IC binding to FcγRI [49] and may explain the clear chondrocyte death seen during IC-mediated arthritis. In the absence of FcγRI, chondrocyte death was completely absent [10, 44] at day 7 after AIA induction. In contrast, FcγR binding may also lead to a rise in inhibitors like TIMPs, which may efficiently reduce MMP-mediated cartilage destruction.

When infiltrating cells are activated by bacterial or yeast cell walls when injected into the knee joint of mice, although a pronounced inflammation developed, MMP-mediated cartilage destruction nor chondrocyte death was detected [27]. Although these cells express an inflammatory phenotype, the released factors were incapable of activating latent MMP which were found in large amounts within the

cartilage layers of the joint [27]. In line with this we now find that in knee joints of arthritic triple KO, despite abundant joint inflammation, no VDIPEN epitopes nor chondrocyte death was observed. Like in the non-IC arthritis, the infiltrated cells despite expressing an inflammatory phenotype were incapable to activate MMPs. This again confirms that FcγR activation is a prerequisite for inducing irreversible cartilage destruction.

The present study underlines that activating FcγR are crucial in induction of severe cartilage destruction and that FcγRII is an important inhibiting receptor which regulates both chronic joint inflammation as well as cartilage destruction during arthritis. FcγRII may be a powerful inhibitor to prevent both synovial inflammation and cartilage destruction and its overexpression may form a new therapeutic tool to combat the severe pathogenicity of ICs involved in RA.

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Chapter 4

Coordinate expression of activating Fcγ receptors I and III and inhibiting Fcγ receptor II in the determination of joint inflammation and cartilage destruction during immune complex-mediated arthritis

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Objective: To study the role of the activating Fcγ receptor types I and III (FcγRI and FcγRIII, respectively), and the inhibiting Fcγ receptor (FcγRII) in inflammation and in various aspects of cartilage destruction during arthritis that is solely induced by immune complexes.
Methods: Immune complex-mediated arthritis (ICA) was passively induced by lysozyme-antilysozyme complexes in FcγRI-, FcγRIII-, and FcγRII-knockout mice and their wild-type controls. Total knee joint sections were isolated to study inflammation and cartilage destruction (loss of proteoglycans (PG), chondrocyte death, matrix metalloproteinase (MMP)-mediated neoepitope expression (VDIPEN), and erosion). The presence of an active phenotype of macrophages was studied by detection of myeloid-related proteins 8 and 14 (MRP8 and MRP14, respectively).
Results: Influx and activation of inflammatory cells (MRP expression) during ICA was decreased in FcγRIII-deficient mice and enhanced in mice lacking FcγRII. Mild cartilage destruction reflected by loss of proteoglycans was consistent with the degree of inflammation. Mice lacking FcγRIII showed almost no PG depletion, whereas in FcγRII$^{-/-}$ mice, PG depletion was increased 3-7- fold in various cartilage areas. Initiation of erosive cartilage destruction, as reflected by MMP-mediated VDIPEN expression was reduced in FcγRIII$^{-/-}$ and FcγRI$^{-/-}$ mice, directing the two different critical steps of cellular influx and subsequent activation. These aspects were enhanced in FcγRII$^{-/-}$ mice. In FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice, VDIPEN expression was 90 – 99% lower, whereas in FcγRII$^{-/-}$ mice, VDIPEN expression was increased 4-fold. Chondrocyte death was reduced in FcγRIII$^{-/-}$ mice (68% lower) and enhanced in FcγRII$^{-/-}$ mice (6-12-fold higher). Progression of arthritis and erosion of the cartilage surface were markedly elevated in FcγRII$^{-/-}$ arthritic joints.
Conclusion: During ICA, FcγRIII is the dominant activating receptor mediating joint inflammation, whereas both FcγRI and FcγRIII are involved in cartilage destruction. FcγRII inhibits both joint inflammation and severe cartilage destruction during IC-mediated arthritis.

IgG-containing immune complexes (ICs) are found in the serum and joint tissue of most patients with rheumatoid arthritis (RA) [1,2] but the role of these complexes in the pathogenicity of this disease is still a matter of debate. ICs can communicate via Fcγ receptors (FcγRs). These receptors are expressed on macrophages residing within the intima layer, which covers the inside of normal diarthrodial joints. Furthermore, they are present on other hematopoietic cells, which infiltrate into the synovium during arthritis [3-5]. In mice, FcγRs are divided into three classes: the high-affinity activating FcγRI (CD64), the low-affinity inhibitory FcγRII (CD32) and the low-affinity activating FcγRIII (CD16)

[6]. FcγRI and FcγRIII are multimeric receptors containing both a ligand-binding α-subunit and the associated signaling subunit, the immunoreceptor tyrosine-based activation motif (ITAM)-containing γ-chain. FcγRII is a single subunit receptor containing the immunoreceptor tyrosine-based inhibitory motif (ITIM) [7]. The latter suppresses B cell, mast cell, neutrophil, and macrophage activation triggered by cross-linking B cell receptor (BCR) or activating FcγRs [8].

During experimental arthritis passively induced by lysozyme-antil-ysozyme ICs (immune complex-mediated arthritis (ICA)), a prominent inflammatory cell mass in the synovial layer and joint cavity and severe cartilage destruction are found [9]. Cartilage destruction starts with proteoglycan (PG) depletion, which is a reversible process, followed by irreversible collagen fiber degradation. The matrix metalloproteinases (MMPs) stromelysin and collagenase, which generate specific cleavage sites within the matrix molecules, are involved in this process [10-12], and blocking of interleukin-1 (IL-1) prevents erosive damage [13]. MMPs are secreted in an inactive form by IL-1-stimulated chondrocytes and stored within the cartilage matrix, and become active after further cleavage [14]. This process is still poorly understood, but MMP activation is primarily found in those arthritis models in which ICs are present and in which synovial lining macrophages are crucial in cartilage pathology [15-17]. The lining macrophages meet ICs formed in the joint cavity and their depletion prevents arthritis. It suggests that the Fcγ receptor balance on the membrane of these cells may determine the severity of inflammation and destruction.

Recently, we found that activating FcγRs play an important role in ICA. After induction of arthritis in the knee joints of FcR γ-chain-deficient mice (which fail to express functional activating FcγR), inflammation and cartilage destruction were completely absent [18]. FcγR dependency in IC-mediated inflammation varies in different body compartments. FcγRIII is the dominant FcγR involved in inflammation in the kidney [19] and in the skin [20], whereas FcγRI is dominant in peritonitis [21]. Type II collagen-induced arthritis (CIA) was absent in FcR γ-chain-deficient mice [22]. Although the γ-chain is not only present in activatory FcγR but also in FcεR, this study strongly indicates that FcγRI and/or III are involved during CIA [22]. Intriguingly, we found in a T cell mediated antigen-induced arthritis model that FcγRI was involved in cartilage destruction but not in joint inflammation [23,24].

To evaluate a distinct role of FcγRI and FcγRIII in joint inflammation and cartilage destruction during arthritis solely induced by ICs, we elicited ICA in the recently generated, selective FcγRI-, FcγRIII-, and FcγRII-deficient mice and their wild-type controls. Joint inflammation and cartilage destruction were investigated by histologic analysis of total knee joints. Macrophages were studied by the expression of myeloid-related proteins 8 and 14 (MRP8 and MRP14, respectively), which are two calcium-binding proteins that characterize a proinflammatory cell population [25,26]. Cartilage destruction was measured by expression of MMP-induced aggrecan neoepitopes (VDIPEN), chondrocyte death, and erosion of the car-

tilage matrix. It was found that FcγRIII determines the inflammation in ICA, whereas both FcγRI and FcγRIII are involved in irreversible cartilage destruction. Moreover, FcγRII inhibits both the severity of inflammation and the destruction of cartilage, suggesting that therapeutic overexpression of FcγRII during arthritis can be protective against development of inflammation and cartilage damage.

Material and Methods

Animals

FcγRI$^{-/-}$ mice, which were lacking the α-chain of FcγRI, were generated (by SV) and were backcrossed to the BALB/c background for 6 generations [27]. FcγRIII$^{-/-}$ mice, which lack the α-chain of FcγRIII (generated by SV), were backcrossed to the C57Bl/6 background for 12 generations [28]. FcγRII$^{-/-}$ mice were developed by Dr. Toshiyuki Takai [29] in the 129 SV (H-2b) and C57Bl/6 (H-2b) background. Control C57Bl/6 and 129SV / C57Bl/6 hybrids were derived from Jackson laboratories (Bar Harbor, ME, USA) and bred in our own facilities. BALB/c mice were obtained from Charles River Institute (Sulzfeld, Germany). Homozygous mutants and their wild-type controls (10-12 weeks old) were used in the experiments. Mice were fed a standard laboratory diet and tapwater ad libitum. Ethical approval was obtained from the local research ethics committee.

Induction of ICA

ICA was passively induced by injecting 3 μg of a cationic antigen (lysozyme coupled to poly-L-lysine) in 6 μl pyrogen-free saline in the knee joints of mice that had previously (16 hours earlier) received, intravenously, polyclonal antibodies directed against lysozyme. The latter antibodies had been raised in rabbits. A moderate arthritis developed in the mice, which was characterized by influx of polymorphonuclear cells, apparent joint swelling, and cartilage destruction as demonstrated by PG depletion and MMP-mediated cartilage damage [9].

Histology of arthritic knee joints

Total knee joints of mice were isolated at days 1, 3, and/or 7 after induction of arthritis. For standard histology, tissue was fixed in 4% formaldehyde, decalcified in formic acid and subsequently dehydrated and embedded in paraffin. Paraffin sections (7 μm thick) were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany).

Hematoxylin and eosin staining was performed to study joint inflammation. Infiltrate and exudate were scored separately. The severity of inflammation in the knee joints was determined using an arbitrary score (0-3). Scoring was performed in a blinded manner by two independent observers (0: no cells, 1: mild cellularity, 2: moderate cellularity, and 3: maximal cellularity).

To study PG depletion from the cartilage matrix, sections were stained with safranin O followed by counterstaining with fast green. Depletion of PG (loss of red staining) from various cartilage layers (patella and femur) was determined using an arbitrary scale from 0-3. Normal cartilage was scored 0, whereas cartilage fully depleted of proteoglycans

was scored the maximum value of 3.

Chondrocyte death was examined by determining the amount of empty lacunae in the cartilage, and results were expressed as a percentage of the total amount of cells (empty lacunae and viable chondrocytes).

Erosion was defined as ruffling of the cartilage surface and expressed as the percentage of impaired cartilage surface of the total cartilage. In all experiments, these variables were scored separately and independently.

Immunohistochemical staining for MRP8 and MRP14

Formalin-fixed sections of knee joints were prepared as described above. Rabbit antisera against recombinant murine MRP8 (anti-MRP8) and MRP14 (anti-MRP14) were produced as described previously [30]. The monospecificity of the antibodies was analyzed by immunoreactivity against recombinant MRP8 and recombinant MRP14 and Western blot analysis of lysates of granulocytes [30]. Sections were stained as described previously [30], using a final antibody concentration of 1 μg/ml. Primary antibodies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Isotype-matched antibodies without relevant specificity were used as negative controls (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany). The presence of MRP8- and MRP14-positive cells present in the joint cavity and synovial lining was determined and expressed as a percentage of the total cell population, using an arbitrary score (1: 1 – 30%, 2: 31 – 70%, 3: 71 – 100%).

Immunohistochemical VDIPEN staining

Sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml in 0.1 M Tris HCl, pH 8.0; Sigma, Zwijndrecht, The Netherlands) for 2 hours at 37°C to degrade hyaluronan and remove chondroitin sulfate from the PGs. Subsequently, sections were treated with 1% H_2O_2 in methanol for 20 minutes and with 0.1 % Triton X-100 in phosphate buffered saline for 5 minutes. After being incubated with 1.5 % normal goat serum for 20 minutes, the sections were incubated overnight at room temperature with affinity-purified rabbit anti-VDIPEN IgG. This antibody has been extensively characterised [31-33]. The anti-VDIPEN antiserum detects the VDIPEN C-terminal neoepitope of aggrecan generated by MMPs. As a control, affinity-purified rabbit IgG was used. In addition, sections were incubated with biotinylated goat anti-rabbit IgG, the binding of which was detected with biotin-streptavidin-peroxidase staining (Elite kit; Vector, Burlingame, CA). Induction of the peroxidase staining was detected using nickel enhancement. Counterstaining was performed with orange G (2%). Areas of immunostaining were expressed as a percentage of the total cartilage surface.

Statistical analysis

Significance was tested using the Wilcoxon rank test. P values less than 0.05 were considered significant. Results are expressed as the mean ± SD.

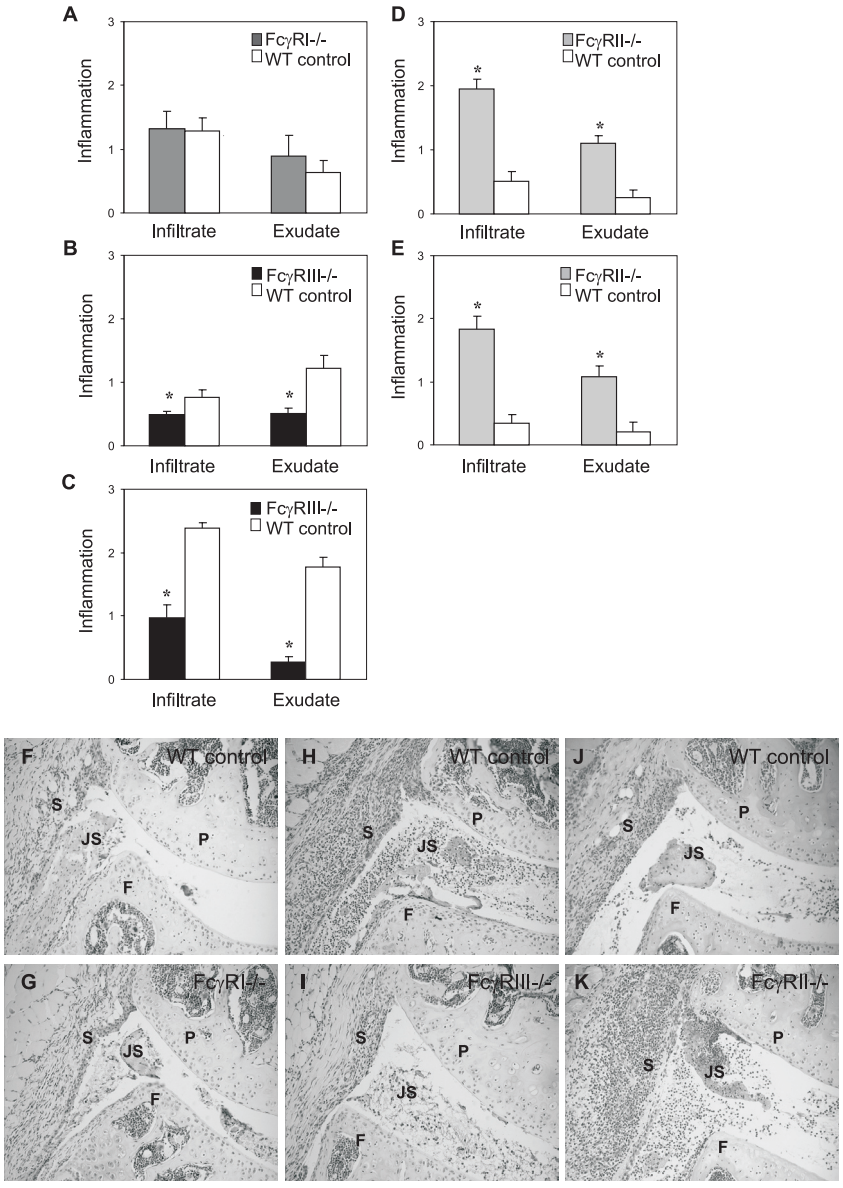

Figure 1

Cellular infiltration (arbitrarily scored 0 – 3, with 3 being maximal cellularity) in frontal sections of whole knee joints in Fcγ receptor I-, II-, and III-deficient ($Fc\gamma RI^{-/-}$, $Fc\gamma RII^{-/-}$, and $Fc\gamma RIII^{-/-}$, respectively) mice and their wild-type (WT) controls at various days after immune complex-mediated arthritis (ICA) induction. Bars show the mean and SD of 10 animals for $Fc\gamma RI^{-/-}$ at day 3 (A), $Fc\gamma RIII^{-/-}$ at day 1 (B) and at day 3 (C), and $Fc\gamma RII^{-/-}$ at day 3 (D) and day 7 (E). Note the significantly lower infiltrate and exudate in $Fc\gamma RIII^{-/-}$ both at day 1 (B) and at day 3 (C) versus WT controls (also in I versus H), whereas comparable cell mass was found in $Fc\gamma RI^{-/-}$ and WT controls at day 3 after ICA induction (A and in G versus F). Significantly higher infiltrate and exudate were found in $Fc\gamma RII^{-/-}$ versus WT controls at day 3 (D and in K versus J) and at day 7 (E) after ICA induction. * = $P < 0.05$ versus WT controls, using the Wilcoxon rank test. P = patella; F = femur, S = synovial lining, JS = joint space. (Original magnification x 100 in F-K).

Results

Determination of joint inflammation of ICA by FcγRIII

Recently, we found that mice lacking both of the functional activating receptors FcγRI and FcγRIII failed to develop synovial inflammation after induction of the passively induced ICA [18]. To investigate the relative role of the two activating receptors, ICA was induced in knee joints of recently generated, selective FcγRI$^{-/-}$ or FcγRIII$^{-/-}$ mice, and their wild-type controls.

Total knee joint sections obtained 3 days after ICA induction showed mild inflammation (both infiltrate in the synovium and exudate in the joint cavity) in FcγRI$^{-/-}$ mice, which did not differ from that in their wild-type controls (Figure 1A, 1F, and 1G).

Interestingly, when ICA was induced in knee joints of FcγRIII$^{-/-}$ mice, markedly less cell influx was evident at day 3 in the synovial layer and joint cavity compared with that in their wild-type controls (Figure 1C, 1H, and 1I). This difference was already apparent at day 1 (Figure 1B), suggesting that, in addition to the role of complement, FcγRIII plays a crucial role not only in the sustained inflammatory response, but also in the early onset of ICA.

In addition to cellular infiltration, joint swelling was measured as the extent of cellular activation, using ^{99m}Tc uptake. A significant reduction in joint swelling was evident in FcγRIII$^{-/-}$ arthritic joints, both at day 1 (mean ± SD ratio of right paw to left paw measurements (R/L), wild-type controls 1.27 ± 0.01 versus FcγRIII$^{-/-}$ mice 1.17 ± 0.03) and at day 3 (R/L ratio wild-type controls 1.20 ± 0.04 versus FcγRIII$^{-/-}$ mice 1.05 ± 0.01).

Enhancement of ICA by FcγRII deficiency

Earlier studies revealed that cross-linking of the inhibiting FcγRII to the activating FcγRs inhibits the intracellular signaling, thereby inhibiting cell activation [7]. Our results show, for the first time, that this inhibition also occurs in vivo in passive ICA. In wild-type controls, a moderate joint inflammation was observed at day 3 (Figure 1D and 1J), which waned rapidly, and at day 7, hardly any inflammatory cells were found (Figure 1E). In contrast, FcγRII$^{-/-}$ mice showed a significantly enhanced joint inflammation at day 3, with 300% more infiltrated cells than their wild-type controls (Figure 1D and 1K). Moreover, inflammation did not wane and remained as severe until day 7 (Figure 1E).

Control studies

A complicating observation was the large variability in severity of ICA in the various wild-type controls, which unfortunately were not in a fully identical genetic background. Earlier work identified major differences in the levels of FcγR expression in closely related mouse strains, which probably underlie these variations. Although the findings in the various knockout were all relative to their genetically identical wild-type controls, it was reassuring to note that we did not find any differences in arthritis between the knockout mice and controls when zymosan was injected in the joint as a general inflammatory trigger (data not

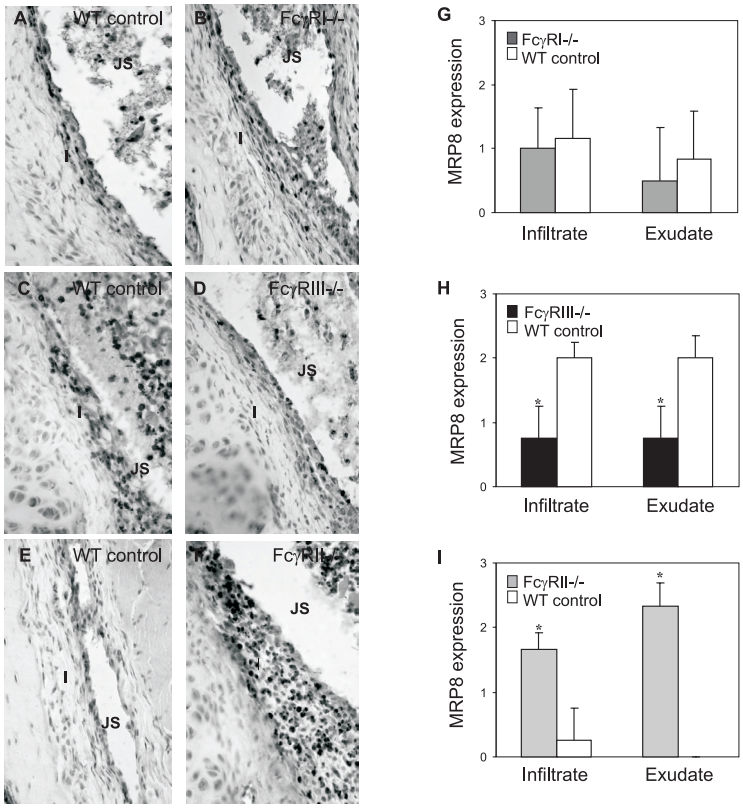

Figure 2

Localization of myeloid-related protein 8 (MRP8) in the synovial lining and joint cavity at day 3 after ICA induction in FcγRI$^{-/-}$ (B), FcγRIII$^{-/-}$ (D), and FcγRII$^{-/-}$ (F) mice versus their wildtype controls (A, C, and E, respectively). Note the significantly lower MRP8 expression in FcγRIII$^{-/-}$ in the synovial lining (D versus C) and in the joint cavity (H) compared with WT controls, whereas similar expression was found in FcγRI$^{-/-}$ and controls (B versus A, and in G). Significantly higher MRP8 expression was found in FcγRII$^{-/-}$ versus controls both in the synovial lining (F versus E) and joint cavity (I). Bars show the mean and SD of 10 mice. * = $P < 0.05$, using the Wilcoxon rank test. I = intima lining (see Figure 1 for other definitions). (Original magnification x 400 in A-F).

shown). This underlines the specificity of the differences noted for ICA.

Differentiation of macrophages to an active inflammatory phenotype antagonistically modulated by FcγRIII and FcγRII

Apart from the amount of infiltrating cells in the synovial tissue, the activation of infiltrating and resident cells and release of mediators will determine the net effect on joint tissues. To investigate the activation state of the resident intima macrophages and the infiltrated cells, we used MRP8 and MRP14 as markers [25,26,30]. MRP8- and MRP14-expressing macrophages are generally found at inflamed sites, and the markers are associated with production of IL-1 and oxidative radicals [34,35].

First, we focused on the synovial lining. Intima cells of FcγRI$^{-/-}$ mice showed expression of MRP8 comparable with that in their controls (Figure 2A and 2B). How-

Table 1 Fcγ receptors (FcγRs) and loss of proteoglycans (PG) during ICA

	ICA day 1		ICA day 3		ICA day 7	
	Patella	Femur	Patella	Femur	Patella	Femur
FcγRI$^{-/-}$	ND	ND	1.3 ± 0.3	2.1 ± 0.3	ND	ND
WT control	ND	ND	0.9 ± 0.2	1.8 ± 0.3	ND	ND
FcγRIII$^{-/-}$	0.3 ± 0.1*	0.5 ± 0.1*	0.3 ± 0.1*	0.4 ± 0.2*	ND	ND
WT control	0.9 ± 0.1	1.6 ± 0.4	1.8 ± 0.2	2.5 ± 0.2	ND	ND
FcγRII$^{-/-}$	ND	ND	2.0 ± 0.4*	2.4 ± 0.4*	1.7 ± 0.3*	2.6 ± 0.4*
WT control	ND	ND	0	0	0.3 ± 0.2	0.7 ± 0.3

Values are the mean ± SD of 10 mice. Loss of red staining, which is a measure of PG depletion, was determined in knee joints at day 1, day 3, and day 7 after induction of immune complex-mediated arthritis (ICA) in FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice, and in wild-type (WT) control mice. Loss of red staining was scored by two blinded and independent observers, using an arbitrary scale of 0-3. (0 = no staining, 1 = minor, 2 = moderate, 3 = maximal). ND = not determined.
* $P < 0.05$ versus controls, by Wilcoxon rank test.

ever, the intima layer of FcγRIII$^{-/-}$ and FcγRII$^{-/-}$ mice showed a much lower (Figure 2C and 2D, respectively, for FcγRIII$^{-/-}$) and higher (Figure 2E and 2F, respectively, for FcγRII$^{-/-}$) number of MRP8-positive intima lining cells compared with their wild-type controls.

In addition, we investigated whether the infiltrated cells exhibited a proinflammatory phenotype. FcγRI$^{-/-}$ mice and wild-type controls showed a comparable expression of MRP8 (Figure 2G), whereas the percentage of infiltrated cells found in FcγRIII$^{-/-}$ knee joints expressing MRP8 was 2 times lower compared with that in their wild-type controls (Figure 2H). In mice that lacked FcγRII, infiltrated cells showed a 5 times higher expression of MRP8 compared with that in their wild-type controls (Figure 2I). MRP14 expression on cells in the synovial lining and joint tissue was identical with MRP8 expression and followed the same pattern as described above in the various knockout mice (data not shown).

Table 2 Fcγ receptors and matrix metalloproteinase activity in the lateral femur during ICA

	VDIPEN expression (% of cartilage surface)
FcγRI$^{-/-}$	0.6 ± 0.4*
WT control	6.1 ± 2.7
FcγRIII$^{-/-}$	0.2 ± 0.2*
WT control	20.0 ± 8.1
FcγRII$^{-/-}$	51.3 ± 4.9*
WT control	12.6 ± 6.3

Values are the mean ± SD percentage of the total cartilage surface of 10 mice, determined as VDIPEN expression in the femoral cartilage surface 3 days after ICA induction in FcγRI$^{-/-}$, FcγRIII$^{-/-}$, and FcγRII$^{-/-}$ mice, and in WT control mice. The percentage of immunostained cartilage surface was determined by two blinded and independent observers. * $P < 0.05$, by Wilcoxon rank test.

Involvement of FcγRII and FcγRIII in early PG breakdown during ICA

Breakdown of PGs from the cartilage layer characterizes the early phase of cartilage destruction in ICA. Loss of red staining, reflecting PG depletion, was found to be similar in arthritic knee joints of FcγRI$^{-/-}$ mice and their wild-type con-

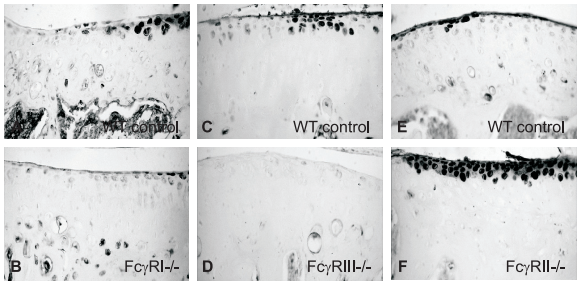

Figure 3
VDIPEN expression in the femoral region of FcγRI$^{-/-}$ (B), FcγRIII$^{-/-}$(D), and FcγRII$^{-/-}$(F) mice versus their WT controls (A, C, and E, respectively). VDIPEN expression was significantly lower in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$, and much higher in FcγRII$^{-/-}$ mice, compared to their wildtype controls. See Figure 1 for definitions. (Original magnification x 250).

trols (Table 1). In contrast, PG loss was significantly lower both at day 1 and day 3 after ICA induction in FcγRIII$^{-/-}$ mice compared with their wild-type controls (Table 1). Nearly maximal loss of red staining was found in FcγRII$^{-/-}$ mice at day 3, whereas their wild-type controls showed only limited PG depletion (Table 1). Almost maximal PG depletion was still present at day 7 after ICA induction in these mice (Table 1), consistent with the prolonged presence of active inflammation.

Effect of FcγRI, -II, and -III on MMP-mediated cartilage damage, chondrocyte death, and cartilage erosion

Shortly after the initial PG loss, MMPs become involved in further degradation of aggrecan and type II collagen, eventually leading to irreversible cartilage destruction [11]. Degradation of aggrecan by MMPs leaves a specific neoepitope in the cartilage that ends on the amino acid sequence VDIPEN, which can be detected by specific antibodies.

In arthritic knee joints of wild-type control mice, VDIPEN neoepitopes were induced mainly at the margins of the femoral-tibial region. VDIPEN was most prominently present in the lateral femur, which is the cartilage surface we focused on. Of extreme interest, although PG loss did not differ between FcγRI$^{-/-}$ mice and their controls, these mice showed a 90% decrease in VDIPEN expression (Table 2, Figure 3A and 3B). VDIPEN expression was also reduced by 99% in FcγRIII$^{-/-}$ mice (Table 2, Figure 3C and 3D), whereas FcγRII$^{-/-}$ mice showed a 4-fold increase in MMP mediated breakdown compared with their wild-type controls (Table 2, Figure 3E and 3F).

As a result of MMP activation, irrevers-

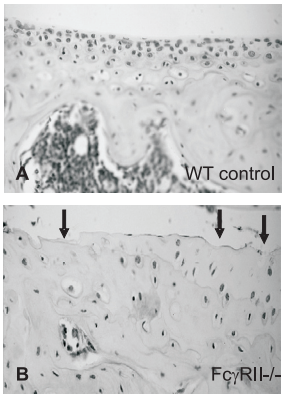

Figure 4
Erosion and chondrocyte death in the femoral region of FcγRII$^{-/-}$ mice (B) and their WT controls (A). Note the ruffled surface of the cartilage (arrows) and large amounts of empty lacunae representing chondrocyte death. See Figure 1 for definitions. (Original magnification x 250).

ible cartilage destruction (chondrocyte death and matrix erosion) may develop. The percentage of chondrocyte death was evaluated in patellar and femural cartilage surface of arthritic knee joints. Chondrocyte death was almost absent both in the FcγRI$^{-/-}$ mice and in their wild-type controls, thus not providing a sufficient window for evaluation. Chondrocyte death in FcγRIII$^{-/-}$ mice was low and comparable with that in their controls 1 day after ICA induction. Three days after ICA induction, the percentage chondrocyte death was 3 times lower in the cartilage surface of the femur of FcγRIII$^{-/-}$ mice compared with their controls (FcγRIII$^{-/-}$ 7 ± 1.6 versus wild-type controls 22 ± 4.5), whereas no significant difference was found in the patellar cartilage surface (FcγRIII$^{-/-}$ 5 ± 1.8 versus wild-type controls 8 ± 1.1). In contrast, in the patella and femur cartilage layers of FcγRII$^{-/-}$ mice, chondrocyte death was 6 times higher in the femoral region at day 3 (33 ± 7.5 versus 5 ± 2.8 in controls) and even 12 times higher at day 7 after ICA induction (50 ± 9.3 versus 4 ± 1.6 in controls) (Figure 4A and 4B).

In addition, cartilage erosion, which reflects the ultimate step to irreversible cartilage destruction, was evaluated. As expected at this early time point, no significant erosion could be detected in the cartilage layers of FcγRI$^{-/-}$ or FcγRIII$^{-/-}$ mice and their wild-type controls. However, in arthritic FcγRII$^{-/-}$ knee joints, significant erosion was observed at day 3 (patella 3 ± 1.2 and femur 5 ± 1.6) and day 7 (patella 4 ± 1.5 and femur 9 ± 2.8) of ICA, whereas erosion was absent in cartilage surfaces of wild-type control mice (Figure 4A and 4B). This identifies FcγRII as a suppressive regulator of severe cartilage destruction.

Discussion

In the present study, we find that ICA induced within the murine knee joint is predominantly mediated by FcγRIII, whereas erosive cartilage damage is mediated by both FcγRI and FcγRIII. We also find that FcγRII is an efficient inhibitor of both acute inflammation and cartilage destruction.

In this study, we used an arthritis model mediated solely by ICs. The onset of inflammation is determined by intima macrophages residing in the synovial lining of the joint. Removal of these macrophages prior to induction of ICA largely prevents the onset of inflammation [17]. The severity of this model is influenced by the genetic background of the mice [18], which explains the observed differences in arthritis found among the various wild-type controls of the knockout mice.

We demonstrated that inflammation after induction of ICA is determined by the activating FcγRIII. This important role for FcγRIII in IC-mediated inflammation was also found in the kidney [19], the lung [36] and the skin [20]. Because macrophages are involved in the onset of inflammation, activation of macrophages was studied using MRP8 and MRP14, which are markers for a proinflammatory phenotype of macrophages. These two calcium-binding proteins of the S100-family are generally expressed by macrophages in various inflammatory reactions but not by resting-tissue macrophages [26,30,37]. Our results show that FcγRIII regulates induction of these molecules in macrophages.

Published data indicate a potential role for these molecules in inflammation.

MRP8- and MRP14-expressing macrophages have been shown to be the major source of TNF-α, IL-1, and respiratory burst under inflammatory conditions in vivo [34,35]. Recent data indicate that MRP8 and MRP14 are expressed in high amounts in synovia of human rheumatoid arthritis and are released at the site of inflammation [38]. They propagate adherence of leukocytes to endothelial cells and exhibit chemotactic activity in vitro. Absence of MRP8 and MRP14 both in the lining cells and in the inflammatory cells in FcγRIII$^{-/-}$ joints suggests that activation of macrophages is clearly mediated by FcγRIII. Increased expression of MRP8 and MRP14 in FcγRII$^{-/-}$ joints indicates that FcγRII inhibits this activation.

An explanation for the FcγRIII dependency found during ICA could be that FcγRIII is highly expressed on macrophages in normal joints, whereas FcγRI expression remains low. FcγR expression within the joint may be altered by local production of cytokines. In human monocytes, FcγRI is up-regulated by interferon-γ (IFN-γ) [39], granulocyte colony-stimulating factor (G-CSF) [40], and IL-10 [41], whereas transforming growth factor β (TGFβ) is potent in up-regulation of FcγRIII [42]. Moreover, other cytokines, such as IL-4 and IL-13, down-regulate activating FcγR [39]. Alteration of the FcγRIII dependency occurs during T cell-mediated antigen-induced arthritis, which is not blocked in FcγRIII$^{-/-}$ mice[23,24]. This might be explained by the fact that T cell-derived cytokines, such as IFN-γ produced by activated T cells, induce up-regulation of FcγRI, resulting in a shift of the involvement of FcγRIII toward FcγRI [43,44].

Apart from FcγRs, complement also plays a major role in IC-mediated inflammation, and a codominance between these two factors has been suggested [45]. In our model, complement activation is regulated by the classical complement pathway, and in earlier studies we found that eliminating C5a, using cobra venom factor, completely abrogated ICA [9], indicating an important role for complement. The FcγRIII and complement C5a dependency was also found in the novel glucose-6-phosphate isomerase (GPI) ICA model [46,47]. In this model, arthritis is induced by transfer of K/BxN antibodies directed against the enzyme GPI. However, in contrast to our model, the K/BxN model is mediated by IgG1 isotype antibodies, which do not bind C1q, resulting in activation of complement by the alternative pathway [47]. Both models show that FcγRIII and C5a, elicited by either the classical or the alternative pathway, need to cooperate to elicit optimal joint inflammation.

Absence of FcγRII aggravates joint inflammation after induction of ICA. Stimulation of FcγRII and co-crosslinking with an ITAM-bearing receptor lead to phosphorylation of the tyrosine residue in the ITIM motif. Phosphorylation of this residue leads to the recruitment of SH2-containing phosphatases, which are responsible for dephosphorylation of specific protein tyrosine kinases [48], resulting in deactivation of the cell. Interestingly, in the K/BxN model FcγRII was not involved in inflammation [49]. A clear explanation for this difference in FcγR dependency is not available yet, but subtle differences in mouse strains housed at various sites are often noted. Of interest,

mouse strains sensitive to CIA develop a severe and chronic arthritis after induction of ICA, whereas the same amounts of IC injected into nonsusceptible strains induce only mild arthritis, which wanes after 4 days. This susceptibility is due to differences in the FcγR balance in these mice [50]. Another explanation for the difference in FcγR dependency may be that IgG1 ICs, which are dominant in the GPI model, are less able to cross-link between FcγRIII and II, which is needed for inhibition of FcγRIII by FcγRII. Moreover, our IC model is macrophage mediated, whereas the K/BxN model seems to be dependent on polymorph nuclear cells [46].

During ICA, activated inflammatory cells that migrate into the knee joint are crucial in the destruction of cartilage [51,52]. This destruction can be divided into two phases. The early phase, characterized by breakdown of PGs, is mediated by aggrecanase, a member of the ADAMS (a desintegrin and metalloproteinase) family [11,53]. The second phase is characterized by MMP-mediated matrix destruction, which results in irreversible cartilage erosion [11,12]. PG depletion was lower in FcγRIII$^{-/-}$ mice and higher in FcγRII$^{-/-}$ mice, compared with their wild-type controls, which implies a role for these receptors in reversible cartilage damage and release of aggrecanase. Moreover, FcγRIII and FcγRII modulate the expression of MRP8 and MRP14. MRP8- and MRP14-expressing macrophages are found to be the major producers of IL-1 and oxygen radicals [34;35]. These results suggest that the balance between FcγRIII and FcγRII on inflammatory cells determines their activation state and thereby the release of cytokines and enzymes involved in PG depletion.

Irreversible cartilage destruction is mediated by MMPs, which are released in the cartilage matrix in an inactive form, and major involvement of MMP-3 (stromelysin) and MMP-13 (collagenase) was demonstrated [10-13]. Regulation of these MMPs is complex and is most likely mediated by activated macrophages. Three levels of regulation can be defined: production and secretion of latent MMPs, activation of latent MMPs, and inhibition of activated MMPs. IL-1 has been shown to be the dominant cytokine regulating production of latent MMPs by the chondrocyte [54]. VDIPEN expression was completely prevented after blockage of IL-1 with either anti-IL-1 antibodies [55] or with IL-1 receptor antagonist [13]. Because macrophages are the dominant IL-1-producing cells, FcγR expression on these cells may determine the amount of cytokine released during ICA. Macrophages deficient in either FcγRIII and FcγRII, produced lower and higher levels of IL-1, respectively, after IC stimulation [8].

Formation of active MMP in the cartilage matrix by splitting the signal peptide of latent MMPs is probably regulated by enzymes released from activated phagocytes. The present data and recent work in antigen-induced arthritis [23,24] suggest that activation of macrophages through FcγRI, and not FcγRIII, is crucial in this process. In FcγRI$^{-/-}$ mice, VDIPEN staining was significantly decreased, whereas no differences were found in inflammation and PG loss. Investigations that should identify the distinct enzymes released after FcγRI triggering

are in progress.

The third regulation level is inhibition of MMPs by tissue inhibitors of MMPs (TIMPs). Oxygen radicals are able to dysregulate TIMPs [56]. Activation of FcγR on macrophages induces oxidative burst [57], which results in inhibition of TIMPs [11]. Overkill of the oxydative burst is mediated by FcγRI, which can result in either inhibition of TIMPs or oxygen radicals-mediated chondrocyte death [58]. Activation of FcγRI, therefore, eventually results in irreversible cartilage destruction.

The present study clearly demonstrates that the balance between activatory and inhibitory FcγR determines both the influx and the activation of inflammatory cells and the destruction within a joint, stimulated solely by ICs. During ICA, FcγRIII is dominant in the development of inflammation. Both FcγRI and FcγRIII are important in irreversible cartilage destruction, and the latter receptor acts indirectly by enhancing cell influx and amplification of activated cell mass. The inhibitory FcγRII is the regulating receptor of both inflammation and cartilage destruction and therefore may be an important therapeutic target for ameliorating both processes in the arthritic joint.

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Chapter 5

FcγRI up-regulation induced by local adenoviral-mediated interferon-γ production aggravates chondrocyte death during immune complex-mediated arthritis

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Using various FcγR-deficient mice, we have obtained suggestive evidence that FcγRI on macrophages is responsible for severe cartilage destruction during arthritis mediated by immune complexes (ICs). This role of FcγRI is pronounced in the presence of activated Th1 cells and a likely Th1 cell-derived cytokine mediating up-regulation of FcγRI expression is interferon (IFN)-γ.

We now investigated whether local overexpression of IFN-γ using an adenoviral vector is able to elevate cartilage destruction during experimental immune complex-mediated arthritis (ICA) and to what extent this process is FcγRI-mediated.

IFN-γ overexpression during ICA had no significant effect on the total cell mass infiltrating the knee joint. However, a higher percentage macrophages expressing markers for a proinflammatory phenotype was found and these macrophages were situated in close proximity of the cartilage surface. Interestingly, cartilage destruction as studied by matrix metalloproteinase (MMP)-mediated proteoglycan damage (VDIPEN expression), chondrocyte death, and erosion was significantly increased. This effect of IFN-γ was only found in presence of ICs, as IFN-γ overexpression during zymosan-induced arthritis, which is not IC-dependent, did not lead to severe cartilage destruction. These results imply a crucial role for ICs and the IgG-binding receptors in the aggravation of cartilage damage by IFN-γ.

Local overexpression of IFN-γ induced increased FcγRI mRNA levels in synovium. To study whether this up-regulation of FcγRI mediates aggravation of cartilage destruction, ICA was raised in FcγRI$^{-/-}$ and their wild-type controls. IFN-γ resulted in elevated VDIPEN expression, which was still present in FcγRI$^{-/-}$. Of great interest, chondrocyte death remained low in FcγRI$^{-/-}$.

These results indicate that IFN-γ overexpression deteriorates cartilage destruction in the presence of ICs and that FcγRI is crucial in the development of chondrocyte death.

Rheumatoid arthritis is charactarized by chronic inflammation and cartilage destruction. Macrophages play a key role in the onset and progression of rheumatoid arthritis. Elegant studies performed by Breshnihan and colleagues [1,2] have shown that the abundance and activation of macrophages in the inflamed synovial membrane and pannus correlates closely with the severity of cartilage destruction in rheumatoid arthritis.

Macrophages are present in the synovial intimal layer, which covers the inside of diarthrodial joints. Experimental studies in our laboratory have shown that synovial lining macrophages are involved in onset, propagation, and exacerbation of experimental arthritis mediated by immune complexes (ICs) [3-5].

IgG-containing ICs are abundantly found in rheumatoid arthritis synovium [6] and are thought to be involved in activation of infiltrated and resident hematopoietic cells. ICs can activate macrophages by binding to Fc receptors for IgG (FcγRs) [7,8]. Three classes have been described in the mouse: the high-affinity receptor FcγRI, and the two low-af-

finity receptors FcγRII and FcγRIII [9]. FcγRI and FcγRIII trigger cell activation through a common γ-chain that contains an immunoreceptor tyrosine-based activation motif [10-12]. In contrast, FcγRII contains an immunoreceptor tyrosine-based inhibitory motif that inhibits via co-cross-linking activation signals through immunoreceptor tyrosine-based activation motif-containing receptors [13,14]. Murine macrophages express all three classes of FcγRs.

Recently, we have found that FcγRI is involved in cartilage destruction during experimental arthritis mediated by ICs [15] and this role seemed to be even more pronounced when T cells are also involved, as in the chronic antigen-induced arthritis [16]. The T cell subsets mediating antigen-induced arthritis are not exactly defined yet. However, this model shows similarities with the collagen type II-induced arthritis [17-19], in which Th1 cells are of importance. One of the most characteristic mediators primarely released by Th1 cells is interferon (IFN)-γ. IFN-γ has a wide variety of proinflammatory actions such as activation of macrophages to produce inflammatory mediators and promoting the killing of intracellular organisms [20-22]. IFN-γ is also known to induce a marked up-regulation of FcγRI expression [23-25].

In the present study we investigated whether local overexpression of IFN-γ using an adenoviral vector aggravates cartilage destruction in a FcγRI-dependent manner. Local overexpression of IFN-γ induced only deterioration of cartilage destruction during immune complex-mediated arthritis (ICA), whereas no effects were found when IFN-γ was overexpressed during zymosan-induced arthritis (ZIA), which is an IC-independent model. As IFN-γ is able to up-regulate FcγRI, FcγRI mRNA levels were detected in synovium. An increase of FcγRI mRNA levels was found and to define the role of FcγRI in the deterioration of cartilage destruction when IFN-γ was overexpressed, we used selective FcγRI-deficient mice. Our findings indicate that local overexpression of IFN-γ aggravates cartilage destruction only in presence of ICs, and that chondrocyte death is mediated by FcγRI-dependent processes.

Material and Methods

Animals

C57Bl/6 mice were purchased from Charles River Lab (Sulzfeld, Germany). FcγRI$^{-/-}$ mice (Dr Verbeek) were backcrossed to the BALB/c background for 6 generations [26]. Homozygous mutants and their wild-type controls (10-12 weeks old) were used in the experiments. Mice were fed a standard diet and tapwater ad libitum.

Overexpression of IFN-γ in vivo using an adenovirus

The recombinant adenovirus-encoding murine IFN-γ (AdIFN-γ) was generated as described before [27]. As control adenovirus AdeGFP, encoding green fluorescent protein, was used. Knee joints of naive mice were intra-articularly injected with 6 μl phosphate-buffered saline (PBS) or with 6 μl of either AdIFN-γ or AdeGFP (1.10^7 pfu). At different time points, patellae with adjacent synovium were dissected in a standardized manner

[28] and synovium biopsies were taken using a biopsy punch with a diameter of 3 mm. Total RNA was extracted in 1 ml TRIzol reagent [29] (Invitrogen, Carlsbad, CA) and used for quantitative polymerase chain reaction (PCR) as described below. PBS, AdIFN-γ, or AdeGFP were intra-articularly injected one day before arthritis induction.

Induction of immune complex-mediated and zymosan-mediated arthritis

Polyclonal antibodies directed against lysozyme were intravenously injected into mice. These antibodies were raised in rabbits. ICA was then induced by injecting 3 μg of PLL-lysozym in 6 μl pyrogen-free saline into the right knee joint. Zymosan arthritis was induced by injecting 180 μg sterilized zymosan into the right knee joint.

Joint swelling

Joint swelling was determined by ^{99m}Tc uptake measurements of the knee joint at days 1 and 3 after arthritis induction [30]. Briefly, mice were injected with 12 μCi of ^{99m}Tc and sedated with 4.5% chloral hydrate. After 30 minutes, the amount of radioactivity was determined by external gamma counting. Arthritis was scored as the ratio of ^{99m}Tc uptake in the right (R) and the left (L) knee joint. R/L ratios > 1.1 were taken to indicate significant swelling of the right knee.

Histology of arthritic knee joints

Total knee joints of mice were isolated at day 3 after induction of arthritis. For standard histology, joints were decalcified, dehydrated, and embedded in paraffin. Sections of 7 μm thick were made and stained with hematoxylin and eosin. Serial sections were scored by two observers on decoded slides. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity. Chondrocyte death was scored as the amount of empty lacunae expressed as percentage of total amount of cells within the cartilage layers. Cartilage erosion was scored by expressing the amount of eroded cartilage as percentage of the total cartilage surface. Chondrocyte death and erosion were determined using cartilage surfaces of the lateral femur-tibia, and medial femur-tibia and data shown are the mean chondrocyte death and erosion expression present in these cartilage layers.

Immunohistochemical staining of polymorphonuclear cells (PMNs)

Sections were stained as described earlier using NIMP-R14, a specific rat anti-mouse PMN monoclonal (diluted 1:50) [3]. Primary antibodies were detected using rabbit anti-rat peroxidase. Finally, sections were counterstained with hematoxylin. The percentage of PMNs was determined in two representative locations of the synovial lining and joint cavity. A total of 100 cells was counted and the amount of brown-stained cells was expressed as percentage PMNs of the total cell population.

Immunohistochemical staining of Myeloid-Related Proteins (MRP)8 and 14

Sections were stained as described earlier using a final antibody concentration of 1 μg/ml [31-33]. Primary antibodies

were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's hematoxylin (Merck, Germany). The percentage of MRP8- and MRP14-positive cells was determined in two representative locations of the synovial lining and joint cavity. A total of 100 cells was counted and the amount of red-stained cells was expressed as percentage activated macrophages of the total cell population.

Immunohistochemical VDIPEN staining

Sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml in 0.1 M Tris HCl, pH 8.0; Sigma, Zwijndrecht, The Netherlands) to remove the side chains of PGs. Subsequently, sections were treated with 1% H_2O_2, 1.5% normal goat serum, and affinity-purified rabbit anti-VDIPEN IgG [34-36]. Thereafter, sections were incubated with biotinylated goat anti-rabbit IgG, and avidin-streptravidin-peroxidase (Elite kit; Vector, Burlingame, CA). Development of the peroxidase staining was performed. Counterstaining was done with orange G (2%). Areas of immunostaining were expressed as percentage of the total cartilage surface. The cartilage layers of the lateral femur-tibia and medial femur-tibia were used to determine the percentage VDIPEN expression and data shown are the mean of these cartilage surfaces.

Measurement of IFN-γ by Enzyme-Linked Immunosorbent Assay

To determine the levels of IFN-γ in washouts, patellae with adjacent synovium were isolated in a standard manner and incubated in RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) for 1 hour at room temperature. IFN-γ levels in the supernatants were measured using a specific sandwich enzyme-linked immunosorbent assay. The capture antibody, monoclonal rat anti-mouse IFN-γ (Pharmingen, San Diego, CA), was coated overnight in a 96-well plate. After incubating with the supernatants, wells were washed three times and incubated using a biotinylated antibody, rat anti-mouse IFN-γ. The second antibody was detected using poly-horseradish peroxidase and subsequently developed using 3, 3, 5, 5 – tetramethylbenzidine/ureum peroxidase solution. Absorbance was measured at 492 nm. The cytokine concentration in the samples was calculated as pg/ml using recombinant murine IFN-γ as standard in the calibration curve.

Determination of MIP-1α and KC levels

To determine levels of KC and MIP-1α in patellae washouts, patellae were isolated in a standard manner and incubated in RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) for 1 hour at room temperature. Chemokine levels were determined using the BioPlex system from BioRad (Hercules, CA) in combination with multiplex cytokine and chemokine kits for the Luminex multianalyte system.

Quantitative detection of FcγRI mRNA using Reverse Transcriptase-PCR

Specific mRNA level for FcγRI was quantified using the ABI/PRISM 7000 Sequence Detection System (ABI/PE,

Table 1. Primers for detection of murine FcγRI mRNA.

	Primer Sequence (5' - 3')
GAPDH	
Up	GGCAAATTCAACGGCACA
Low	GTTAGTGGGGTCTCGCTCCTG
FcγRI	
Up	ACACAATGGTTTATCAACGGAACA
Low	TGGCCTCTGGGATGCTATAACT

Primer sequences for quantitative PCR on synovium samples.

Foster City, CA). Briefly, 1 μg of synovial RNA was used for reverse transcriptase-PCR. mRNA was reverse-transcribed to cDNA using oligodT primers and 1/20 of the cDNA was used in one PCR amplification. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C with data collection in the last 30 seconds. Message for murine GAPDH and FcγRI was amplified using specific primers (Biolegio, Malden, The Netherlands) for GAPDH and FcγRI (Table 1) at a final concentration of 300 nmol/L. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the FcγRI gene in the different samples after correction of the GAPDH content for each individual sample to rule out confounding by variation of the RNA purification and reverse transcriptase step.

Statistical analysis

Differences between experimental groups were tested for significance using the Mann-Whitney U-test with the statistic program GraphPad Prism 3.0. P values less than 0.05 were considered significant.

Results

Kinetics of IFN-γ overexpression in naive knee joints

AdIFN-γ was injected into the right knee joint of naive mice and subsequently IFN-γ levels were measured in washouts from synovium specimen taken at 6, 24, 48, and 72 hours after injection. IFN-γ was already detected at 6 hours (1230 pg/ml), reached its maximum at 24 hours (2870 pg/ml), and waned thereafter. After 48 hours, IFN-γ was below detection level. Control eGFP adenoviral vector did not induce IFN-γ production.

Because IFN-γ is a proinflammatory cytokine, this short-lasting IFN-γ production within the joint may on itself lead to inflammation. Using ^{99m}Tc uptake, no swelling was measured in knee joints that received AdIFN-γ or AdeGFP (data not shown). Histology of total knee joint sections showed no inflammatory cell mass in the joint cavity and only a mild activation of the synovial lining was induced by both adenoviruses (Figure 1).

IFN-γ overexpression has no effect on the amount of infiltrating cells, but increases the population of activated macrophages in the joint cavity

To investigate whether local IFN-γ production, in the presence of ICs, leads to enhanced joint inflammation, we injected PBS, AdeGFP, and AdIFN-γ, 1 day before induction of ICA. Injection of either AdIFN-γ or AdeGFP resulted in a 30% decrease in joint swelling, compared to mice that had received PBS (Table 2). Three days after ICA induction, swelling in the AdIFN-γ group and PBS group was

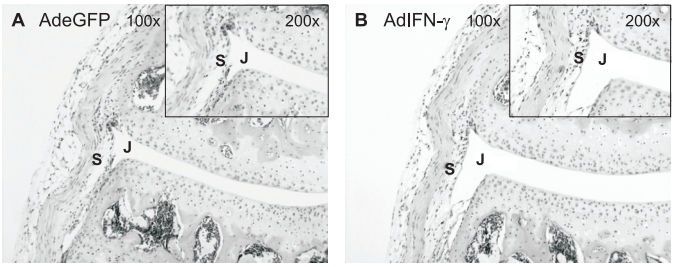

Figure 1
Histology of naive knee joints 24 hours after injection of AdeGFP (A) or AdIFN-γ (B) (1 .10^7 pfu). Injection of both AdeGFP and AdIFN-γ resulted in a mild thickening of the synovial lining (S), whereas no inflammatory cell mass was found in the joint cavity (J). Original magnification, x100 (A,B), x 200 (insert).

comparable (Table 2). The inflammatory cell mass in joint cavity (exudate) and synovium (infiltrate) at day 3, was similar in the PBS, AdeGFP, and AdIFN-γ groups (Table 2).

To study whether IFN-γ overexpression altered the composition of the cell mass, PMNs were stained (Figure 2A). The percentage of PMNs infiltrated in the synovium was similar in the PBS, AdeGFP, and AdIFN-γ group (± 45-50%). Interestingly, in the joint cavity the percentage of infiltrated PMNs was significantly lower in the AdIFN-γ group (50%) compared to the PBS and AdeGFP groups (70%), indicating that macrophages are more abundantly present in the AdIFN-γ group.

As stimulation of macrophages with IFN-γ modulates chemokine production [37, 38], we measured macrophage inflammatory protein (MIP)-1α, which attracts monocytes [39] and KC, a neutrophil attractant [39]. IFN-γ overexpression in naive joints resulted in 220 pg/ml MIP-1α levels after 3 days, injection of AdeGFP induced only 25 pg/ml. KC levels were not up-regulated by IFN-γ (30 pg/ml), and were similar as found after AdeGFP injection (20 pg/ml). These enhanced MIP-1α levels might explain the increase of infiltrating macrophages found.

Furthermore, we studied the activation state of macrophages using MRP8 and 14 as markers. These S100 proteins are associated with an activated phenotype of macrophages present in inflammatory sites. In the synovial layer, the percentage of macrophages expressing MRP8 was

Table 2 Inflammatory response 1 and 3 days after ICA induction.

	Joint Swelling		Inflammatory cell mass	
	Day 1	Day 3	Joint cavity	Synovium
ICA + PBS	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
ICA + AdeGFP	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
ICA + AdIFN-γ	1.2 ± 0.1	1.3 ± 0.1	1.6 ± 0.2	1.6 ± 0.2

Inflammation was determined using joint swelling and inflammatory cell mass in the joint cavity and synovium. Joint swelling of the right inflamed knee joint was quantified using the right/left ratio of ^{99m}Tc uptake in both knee joints. The amount of inflammatory cells in the joint cavity (exudate) and in the synovial layer (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0-3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that both joint swelling and inflammatory cell mass were not significantly different between the PBS, AdeGFP, and AdIFN-γ group. Data are the mean ± SEM of 12 mice and significance was evaluated using the Mann-Whitney U test (*, P< 0.05).

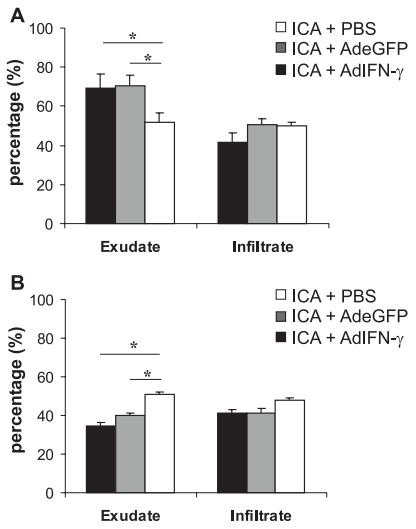

Figure 2
The percentage of PMNs (A) and MRP8-expressing macrophages (B) in the joint cavity (exudate) and synovial lining (infiltrate) 3 days after ICA induction in the PBS, AdeGFP, and AdIFN-γ groups. Note the significantly lower percentage of PMNs and the significant increase of MRP8-expressing macrophages in the exudate of the AdIFN-γ group.Values represent the mean ± SEM of 12 mice. Data were evaluated using the Mann-Whitney U test (*, $P < 0.05$).

similar in all groups (40-45%) (Figure 2B). However, in the joint cavity the percentage MRP8-positive macrophages was somewhat, although significantly, increased in the AdIFN-γ group (50% versus 35-40% in controls) (Figure 2B). Intriguingly, these MRP8-expressing macrophages were clustered in the proximity of the cartilage surface. MRP14 expression on cells in the synovial lining and joint tissue was identical with MRP8 expression and followed the same pattern as described above in all groups (data not shown).

Local overexpression of IFN-γ during ICA results in aggravation of severe cartilage destruction

In addition, we studied the impact of IFN-γ on cartilage destruction in ICA. MMP-mediated cartilage damage (VDIPEN immunostaining), chondrocyte death, and surface erosion were used as histological parameters. Strikingly, injection of AdIFN-γ and subsequent induction of ICA resulted in a two- to three-fold increase in VDIPEN expression in the cartilage matrix, when compared to both control groups (Figure 3, A and D (AdIFN-γ) versus B and C (controls)). Furthermore, chondrocyte death as measured by the percentage of empty lacunae within the cartilage layers was two times higher in presence of IFN-γ, compared to the PBS group (Figure 3, A and G (AdIFN-γ) versus E (PBS)) and even four times elevated when compared to the AdeGFP-injected group (Figure 3, A, G (AdIFN-γ) and F (AdeGFP)).

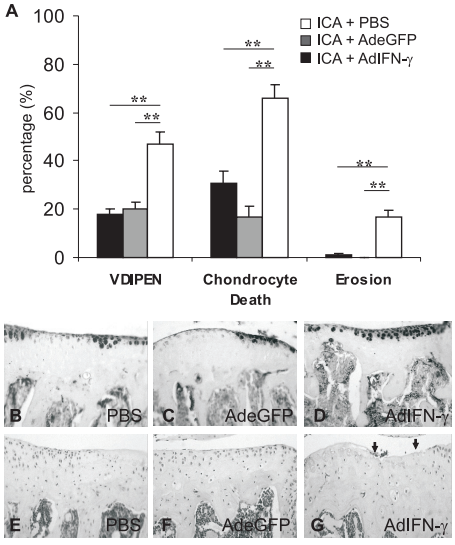

Figure 3
Cartilage destruction, measured as VDIPEN expression (A-D), chondrocyte death, and erosion of the cartilage matrix (A, E-G), in the PBS, AdeGFP, and AdIFN-γ group 3 days after ICA induction. IFN-γ overexpression resulted in a significant increase in VDIPEN expression (A and D versus control B and C), chondrocyte death (A and G versus control E and F), and erosion (A and G (arrows) versus control E and F). Values represent the mean ± SEM of 12 mice. Data were evaluated using the Mann-Whitney U test (**, $P < 0.0001$). Orginal magnification, x 200.

Matrix erosion was determined using the cartilage surfaces of the lateral and medial femur-tibia plateau. Erosion was almost absent in the PBS and AdeGFP group, in line with the early time point chosen (Figure 3, A, E, and F). In contrast, local overexpression of IFN-γ induced erosion, which was found on all cartilage surfaces (Figure 3, A and G). Our results indicate that during an arthritis mediated by ICs, IFN-γ overexpression significantly aggravates irreversible cartilage destruction.

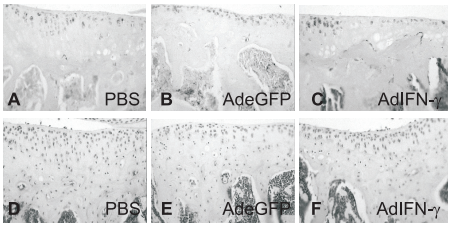

Figure 4
Cartilage destruction after zymosan arthritis. Cartilage destruction, measured as VDIPEN expression (A-C), chondrocyte death, and erosion of the cartilage matrix (D-F), in the PBS, AdeGFP, and AdIFN-γ group, respectively, 3 days after ZIA induction. No VDIPEN expression (C versus control A and B), chondrocyte death and erosion (F versus control D and E) were found. Original magnification, x 200.

Aggravation of cartilage destruction by IFN-γ is IC-dependent

To further investigate whether the aggravating effect of IFN-γ on cartilage destruction is specific for ICs, we also induced zymosan arthritis (ZIA). Twenty-four hours before ZIA induction, mice were injected with either PBS, AdeGFP or AdIFN-γ. IFN-γ resulted in increased number of inflammatory cells in the joint cavity (exudate) at day 3, whereas the infiltrate in the synovium was comparable with both control groups (Table 3). VDIPEN expression, chondrocyte death, and erosion were completely absent in all groups (Figure 4, A to F). These results show that local overexpression of IFN-γ in the knee joint during a non-IC-dependent model, does not elicit irreversible cartilage destruction.

Up-regulation of FcγRI in synovium by IFN-γ

Because we recently found an important role for FcγRI in mediating cartilage destruction, we focused on the expression of this receptor. After injection of AdIFN-γ in knee joints, synovial specimens were

Table 3 **Inflammatory response 3 days after zymosan arthritis induction.**

	Inflammatory cell mass	
	Joint Cavity	Synovium
ZIA + PBS	0.9 ± 0.1	1.0 ± 0.1
ZIA + AdeGFP	1.3 ± 0.2	1.2 ± 0.2
ZIA + AdIFN-γ	1.7 ± 0.1*	0.8 ± 0.2

The amount of inflammatory cells in the joint cavity (exudate) and in the synovium (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0-3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that the inflammatory cell mass is significantly increased in the AdIFN-γ group, when compared to the PBS and AdeGFP group. Data are the mean ± SEM of 8 mice and significance was evaluated using the Mann-Whitney U test (*, P< 0.05).

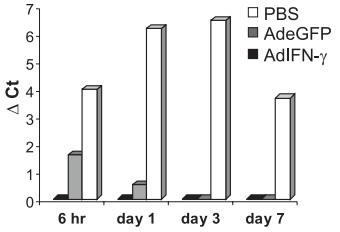

Figure 5
Expression profile of FcγRI mRNA levels after injection of PBS, AdeGFP, and AdIFN-γ in synovium specimens at different time points (6 hrs, day 1, day 3, and day 7). The Ct value of FcγRI in synovium at 0 hours was substracted from the Ct values for FcγRI at different time points after injection. Ct values were corrected for GAPDH content for each individual sample.

isolated at different timepoints (6 hours, 1, 3, and 7 days) and mRNA levels of FcγRI were detected. In naive knee joints FcγRI mRNA could not be detected, whereas 6 hours after AdIFN-γ injection, FcγRI mRNA level was markedly increased (Δ Ct = 3.97). At day 1, maximal values were found, which remained high until day 7 after injection (Figure 5). Injection of PBS had no effect on FcγRI mRNA levels, whereas AdeGFP levels showed an increase at 6 hours (Δ Ct = 1.59), which was decreased at day 1 and completely absent after 3 days (Figure 5).

IFN-γ-induced aggravation of chondrocyte death is FcγRI dependent

As presence of IFN-γ induced an increase of FcγRI mRNA, we further investigated whether this receptor contributed to the aggravation of cartilage destruction using FcγRI-deficient mice and their respective wild-type controls. The IFN-γ effect in these wild-type controls was primarily as expected. FcγRI$^{-/-}$ and wild-type controls received PBS, AdeGFP, or AdIFN-γ, 24 hours before ICA induction. Joint inflammation was similar in both wild-type controls and FcγRI$^{-/-}$ (Table 4). Unexpectedly, IFN-γ enhanced VDIPEN expression was still present in FcγRI$^{-/-}$ (Figure 6A). In the FcγRI$^{-/-}$ related wild-type controls, IFN-γ greatly increased chondrocyte death, resulting in 35% empty lacunae. Of great interest, chondrocyte death remained low in FcγRI$^{-/-}$ (3%) (Figure 6B). Unfortunately, erosion of the cartilage surface could not be detected in these wild-type controls after IFN-γ overexpression, hampering analysis of FcγRI involvement.

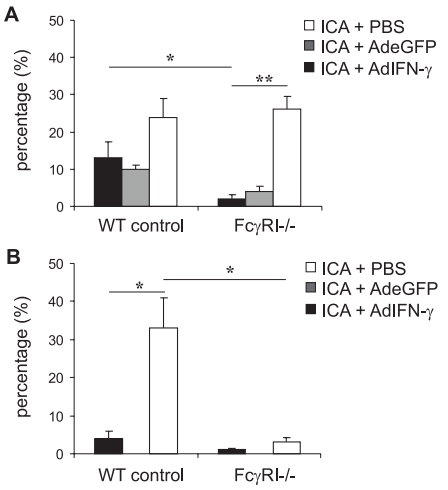

Figure 6
Cartilage destruction, measured as VDIPEN expression (A) and chondrocyte death (B) after injection of PBS, AdeGFP, and AdIFN-γ in FcγRI$^{-/-}$ and their wild-type controls 3 days after ICA induction. Note that IFN-γ resulted in increased VDIPEN expression in both wild-type controls and FcγRI$^{-/-}$. IFN-γ-enhanced chondrocyte death was not found in FcγRI$^{-/-}$. Values represent the mean ± SEM of 6 mice.Data were evaluated using the Mann-Whitney U test (*, P< 0.05, **, P< 0.0001).

Table 4 **Inflammatory response in FcγRI$^{-/-}$ compared to wild-type controls after injection of PBS, AdeGFP, or AdIFN-γ 3 days after ICA induction**

	Inflammatory cell mass	
	Joint Cavity	Synovium
PBS		
WT control	0.7 ± 0.3	1.7 ± 0.3
FcγRI$^{-/-}$	1.1 ± 0.1	1.9 ± 0.3
AdeGFP		
WT control	0.9 ± 0.2	1.6 ± 0.3
FcγRI$^{-/-}$	0.7 ± 0.2	1.6 ± 0.2
AdIFN-γ		
WT control	1.1 ± 0.2	1.7 ± 0.3
FcγRI$^{-/-}$	1.1 ± 0.1	1.3 ± 0.2

The amount of inflammatory cells in the joint cavity (exudate) and in the synovium (infiltrate) were scored in a blinded fashion by two independent observers using an arbitrary scale from 0-3 (0, no; 1, minor; 2, moderate; 3, maximal). Note that the inflammatory cell mass is not significantly different between the PBS, AdeGFP, and AdIFN-γ group. Data are the mean ± SEM of 6 mice and significance was evaluated using the Mann-Whitney U test (*, P< 0.05).

Discussion

In the present study we demonstrate that overexpression of IFN-γ results in increased chondrocyte death by up-regulation of FcγRI. We locally overexpressed IFN-γ in two different arthritis models using an adenoviral vector and found that only in presence of ICs cartilage damage was aggravated. Using FcγRI-deficient mice, it was confirmed that chondrocyte death is mediated by FcγRI and that up-regulation of this receptor by IFN-γ directs deterioration of chondrocyte death.

Injection of AdIFN-γ in naive knee joints induced a short-lasting although high peak of IFN-γ. This might be explained by the ability of IFN-γ to shut off the CMV promotor of the adenovirus [40]. We found that 1 day after injection of AdIFN-γ only a very mild synovial inflammation was induced, indicating that this concentration of IFN-γ was not able to attract large amounts of inflammatory cells. This is in line with a previous study in which recombinant mouse IFN-γ was injected in the peritoneal cavity, and no chemotactic activity for mouse macrophages or neutrophils was found [41]. When triggers such as ICs or zymosan were additionally injected into the joint, a large amount of inflammatory cells infiltrated probably regulated by IL-1 [42] and chemotactic factors [43]. Surprisingly, IFN-γ did not alter the amount of infiltrated cells in ICA, whereas in ZIA the inflammatory cell mass in joint cavity was increased. A possible explanation for this discrepancy might be that IFN-γ elevates joint inflammation dependent on the trigger and receptors involved to induce arthritis. Although AdIFN-γ injection before ICA induction did not contribute to the amount of infiltrated cells, yet the type of infiltrated cells was markedly altered. The percentage of macrophages was significantly higher in the IFN-γ stimulated joint. This is in line with our finding that IFN-γ elevated levels of MIP-1α, a potent chemokine of macrophages, but not of KC, which is dominant for attracting PMNs [39]. Macrophages in IC-mediated arthritis expressed MRP8 and 14, which are markers for a proinflammatory phenotype. MRP8- and MRP14-expressing macrophages have shown to be the major source of IL-1 and respiratory burst under inflammatory conditions in vivo [44,45]. Because the abundance and activation of macrophages is closely correlated to the severity of cartilage destruction [1,2], we further studied this parameter.

IFN-γ may directly stimulate chondrocytes resulting in higher expression of latent stromelysin. Apart from that, expression of aggrecan and other core protein genes can decrease [46-48]. This eventually may lead to enhanced levels of pro-MMPs within the cartilage matrix and decreased proteoglycan synthesis. However, in the present study IFN-γ alone did not induce severe cartilage destruction directly, since injection of AdIFN-γ in naive knee joints failed to induce irreversible cartilage destruction (data not shown) and additional triggers are needed.

When AdIFN-γ was injected before zymosan-induced arthritis, although increased infiltration of inflammatory cells was found, severe cartilage destruction was completely absent. In contrast, when in such an IFN-γ joint, IC-mediated arthritis was induced, a strongly increased MMP-mediated proteoglycan damage,

chondrocyte death, and erosion were found. This suggests an important role for IgG-binding FcγR. In earlier studies using FcγRI$^{-/-}$, we have found that FcγRI is highly involved in mediating severe cartilage destruction during IC-mediated arthritides [15,16]. In the present study, IFN-γ significantly increased FcγRI mRNA levels in the synovium for 7 days which is in line with earlier studies describing up-regulation of FcγRI by IFN-γ [23-25]. This elevated FcγRI expression is exclusively present on the macrophage population, since murine PMNs do not express FcγRI [49]. Unfortunately, it was not possible to detect the murine FcγRI protein as a specific anti-FcγRI antibody is not available. Further proof that elevation of FcγRI induced by IFN-γ is responsible for the observed cartilage destruction was obtained by IFN-γ overexpression in mice lacking FcγRI.

Chondrocyte death appeared to be specifically mediated by FcγRI. Chondrocyte death may be mediated by oxygen radicals. Binding of IgG to FcγRI leads to an overkill of the oxidative burst resulting in prominent oxygen radical production [50]. Apart from that, IFN-γ also regulates the production of nitric-oxide by macrophages [51], which has been shown to induce apoptosis of chondrocytes [52]. The close interaction between macrophages and the cartilage surface we found makes the above mechanisms highly plausible, since oxygen and nitrogen radicals only produce tissue damage within a short distance. Furthermore, the finding that chondrocyte death is absent in FcγRI$^{-/-}$ also indicates that neutrophils are probably not involved, since these cells lack FcγRI [49].

Unexpectedly, we found that IFN-γ still induced cartilage proteoglycan damage mediated by MMPs (VDIPEN epitopes) when FcγRI was absent. One explanation may be that in FcγRI-deficient mice, the other activating FcγRIII is still present and becomes up-regulated after IFN-γ stimulation. As VDIPEN expression was strongly diminished in ICA in both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice, this indicates that also FcγRIII when present in sufficient amounts may significantly contribute to MMP-mediated proteoglycan damage [15].

Eventually, activation of MMPs leads to degradation of the collagen type II network and erosion of the cartilage matrix. In contrast to marked erosion after ICA induction in mice with C57Bl/6 background, erosion was completely absent in the FcγRI$^{-/-}$ but also in their proper controls. As the FcγRI$^{-/-}$ are generated in the Balb/c background and the severity of the ICA model is related to the genetic background of the mice [53], this explains the absence of erosion, but hampers evaluation of this aspect at present.

The present study demonstrates that IFN-γ aggravates irreversible cartilage destruction in the presence of ICs implicating an important role for FcγRI in mediating chondrocyte death. As ICs and macrophages are abundantly found within the synovia of rheumatoid arthritis patients, local production of IFN-γ within the synovium may induce elevated expression of FcγRI on the macrophage which appears to be a crucial receptor involved in mediating severe cartilage destruction. FcγRI may form a new important therapeutic target in order to combat this crippling disease

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Chapter 6

Joint inflammation and chondrocyte death become independent of Fcγ receptor type III by local overexpression of interferon-γ during immune complex-mediated arthritis

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Introduction: Previously, it was shown that the onset and the degree of joint inflammation during immune complex (IC)-mediated arthritis (ICA) depend on FcγRIII. Local adenoviral overexpression of IFN-γ in the knee joint prior to ICA onset aggravated severe cartilage destruction. In FcγRI$^{-/-}$ mice however, chondrocyte death was not enhanced by IFN-γ, whereas metalloproteinase (MMP)-mediated aggrecan breakdown was markedly elevated suggesting a role for the activating FcγRIII in the latter process.
Objective: To study the role of FcγRIII in joint inflammation and severe cartilage destruction in IFN-γ-stimulated ICA using FcγRIII$^{-/-}$ mice.
Methods: FcγRIII$^{-/-}$ and wild-type mice were injected in the knee joint with AdIFN-γ or AdeGFP one day prior to ICA induction. Histology was taken three days after arthritis onset to study inflammation and cartilage damage. MMP-mediated expression of the VDIPEN neoepitope was detected by immunolocalization. Chemokine and FcγR expression levels were determined in respectively synovial washouts and synovium.
Results: Injection of AdIFN-γ in naive knee joints markedly increased mRNA levels of all three FcγRs. Upon IFN-γ overexpression prior to ICA induction, joint inflammation was similar in FcγRIII$^{-/-}$ and WT mice. The percentage of macrophages in the knee joint was increased, which correlated with high concentrations of the macrophage-attractant MIP-1α. Furthermore, IFN-γ induced a 3-fold increase of chondrocyte death in WT controls, which was also present in FcγRIII$^{-/-}$ mice. Remarkably, VDIPEN expression remained also high in FcγRIII$^{-/-}$ mice.
Conclusion: IFN-γ bypasses FcγRIII dependency of the development of ICA. Furthermore, both FcγRI and III can mediate MMP-dependent cartilage matrix destruction.

Rheumatoid arthritis (RA) is a chronic inflammatory synovitis characterized by synovial hypertrophy and synovial pannus formation with accompanying destruction of juxta-articular cartilage and bone [1]. Macrophages play a major role in the arthritic process by releasing multiple factors such as pro-inflammatory cytokines and tissue-degrading enzymes and several studies have shown that the number of macrophages in the joints of RA patients correlates well with joint inflammation [2] and cartilage damage [3,4].

IgG containing immune complexes are abundantly present in the synovium of most RA patients and play a dominant role in the activation of macrophages [5,6]. Fcγ receptors (FcγRs) on macrophages interact with IgG-containing immune complexes [7,8]. These receptors for the Fc portion of the IgG molecule play a central role in immune-mediated tissue injury due to their ability to recruit effector immune cells [9]. Three classes of FcγRs are distinguished on hematopoietic cells: the high affinity receptor

FcγRI (CD64), and the low affinity receptors FcγRII (CD32) and FcγRIII (CD16). FcγRI and FcγRIII are activating receptors, associated with a dimer of a signal transduction subunit the FcR γ-chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM). The single chain FcγRII is an inhibitory receptor, containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain [10].

In a recent study using FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice, we found that during IC-mediated arthritis FcγRIII mainly regulates the inflammatory response, whereas FcγRI is more prominently involved in chondrocyte death and cartilage matrix erosion via activation of matrix metalloproteinases [11,12].

Cartilage damage starts with the reversible process of proteoglycan depletion mediated by aggrecanases. If cartilage destruction continues, irreversible collagen fibre degradation occurs. Stromelysin and collagenase are the main matrix metalloproteinases (MMPs) involved in this process [13-15]. MMPs are secreted in an inactive form by chondrocytes, stored in the cartilage matrix and activated after further cleavage [16]. MMP activation is primarily found when experimental arthritis is elicited by ICs, which suggests an important role for the IC-binding FcγRs in this process.

Cartilage destruction is more pronounced in T cell dependent arthritis models, indicating that Th1 cytokines might be of importance. One of the typical Th1 cytokines secreted by T cells is interferon (IFN)-γ. Local overexpression of IFN-γ during immune complex mediated arthritis (ICA) resulted in more severe cartilage destruction as found in enhanced MMP-mediated proteoglycan (PG) degradation, chondrocyte death, and erosion [17]. In FcγRI deficient mice, chondrocyte death remained low even when IFN-γ was overexpressed, suggesting a crucial role for FcγRI [17]. However, MMP-mediated cartilage destruction was enhanced by IFN-γ in arthritic knee joints of FcγRI$^{-/-}$ mice, indicating that FcγRIII compensates absence of FcγRI.

In the present study, we investigate the particulate role of FcγRIII in joint inflammation and cartilage destruction during IFN-γ-enhanced immune complex arthritis.

This study shows that IFN-γ aggravates MMP-mediated cartilage damage mediated by activating FcγRI and III. Furthermore, we show that both activating FcγRs are redundant in initiating MMP-mediated cartilage destruction, but we confirm a specific role for FcγRI in mediating chondrocyte death.

Material and Methods

Animals

FcγRIII$^{-/-}$ mice, deficient for the α-chain of FcγRIII, were backcrossed to the C57Bl/6 background for 12 generations [18]. Homozygous mutants and their wild-type (WT) controls (10-12 weeks old) were used in the experiments. Mice were fed a standard diet and tapwater ad libitum. Ethical approval was obtained from the local research ethics committee.

In vivo overexpression of IFN-γ using an adenovirus

The recombinant adenovirus encoding murine IFN-γ (AdIFN-γ) was generated as described before [19]. AdeGFP (green fluorescent protein) was used as control virus. Knee joints of naive mice were intra-articularly injected with 6 μl PBS or with 6 μl of either AdIFN-γ or AdeGFP (1.10^7 pfu). At different time points, patellae with adjacent synovium were dissected in a standardized manner [20] and synovium biopsies were taken using a biopsy punch with a diameter of 3 mm. Total RNA was extracted in 1 ml TRIzol reagent and used for quantitative PCR as described below. PBS, AdIFN-γ, or AdeGFP were intra-articularly injected one day prior to arthritis induction.

Induction of immune complex-mediated arthritis

ICA was passively induced by injecting 3 μg PLL-lysozyme in knee joints of mice that had previously (16 hours earlier) received, intravenously, polyclonal antibodies directed against lysozyme. These antibodies were raised in rabbits.

Histology of arthritic knee joints

Total knee joints of mice were isolated 3 days after arthritis onset. Joints were decalcified, dehydrated, and embedded in paraffin. Tissue sections (7 μm) were stained with hematoxylin and eosin. Histopathological changes were scored using the following parameters. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity. Chondrocyte death was scored as the amount of empty lacunae expressed as percentage of total amount of cells within the cartilage layers.

Immunohistochemical detection of macrophage marker F4/80

F4/80, a murine macrophage membrane antigen, was detected using a specific rat anti-mouse F4/80 IgG [21]. Primary antibodies were detected using rabbit anti-rat IgG and avidin-horseradish peroxidase conjugate. Finally, sections were counterstained with haematoxylin. The percentage of macrophages was determined at two representative locations of both the synovial lining and joint cavity in 3 different sections of each knee joint. Using a magnification of 200x, the percentage F4/80-positive cells of the inflammatory cell mass present in the visual field was determined using an arbitrary score from 0-4 (0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%).

Immunohistochemical staining of Myeloid Related Proteins (MRP)8 and 14

Sections were stained as described earlier using a final antibody concentration of 1 μg/ml [22]. Primary antibodies were detected using peroxidase-conjugated second-stage antibodies against rabbit IgG (Dianova, Hamburg, Germany). Finally, sections were counterstained with Mayer's haematoxylin (Merck, Germany).

Immunohistochemical VDIPEN staining

Active MMPs can cleave PGs resulting in the neoepitope VDIPEN, which can be detected by specific monoclonal antibodies. VDIPEN expression indicates presence of active MMPs, which also degrade

collagen fibers resulting in severe cartilage damage. To detect VDIPEN, sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml in 0.1 M Tris HCl, pH 8.0; Sigma, Zwijndrecht, The Netherlands) to remove the side chains of PGs followed by incubation with affinity-purified rabbit anti-VDIPEN IgG [23]. The primary antibody was detected using biotinylated goat anti-rabbit IgG, and avidin-streptravidin-peroxidase (Elite kit; Vector, Burlingame, CA). Counterstaining was done with orange G (2%). Areas of immunostaining were expressed as percentage of the total cartilage surface.

Quantitative detection of FcγRs mRNA using RT-PCR

Specific mRNA-level for FcγRI, II, and III was detected using the ABI/PRISM 7000 Sequence Detection System (ABI/PE, Foster City, CA). Briefly, one μg of synovial RNA was used for RT-PCR. mRNA was reverse transcribed to cDNA using oligodT primers 1/100 of the cDNA was used in one PCR amplification. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C with data collection in the last 30 seconds. Message for murine FcγRI, II, and III was amplified using the following primers (Biolegio, Malden, The Netherlands) at a final concentration of 300 nmol/l: upper 5'-ACA-CAA-TGG-TTT-ATC-AAC-GGA-ACA-3' and lower 5'-TGG-CCT-CTG-GGA-TGC-TAT-AAC-T-3' for FcγRI, upper 5'-GAC-AGC-CGT-GCT-AAA-TCT-TGC-T-3' and lower 5'-GTG-TCA-CCG-TGT-CTT-CCT-TGA-G-3' for FcγRII, upper 5'-GAC-AGG-CAG-AGT-GCA-GCT-CTT-3' and lower 5'-TGT-CTT-CCT-TGA-GCA-CCT-GGA-T-3' for FcγRIII. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the FcγR genes in the different samples after correction of the GAPDH content for each individual sample.

Determination of macrophage inflammatory protein (MIP)-1α and KC levels

To determine levels of KC (chemotactic for PMNs) and MIP-1α (chemotactic for macrophages) in patellae washouts, synovial specimens were isolated in a standard manner, incubated in 200 μl RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) for 1 hour at room temperature and weight hereafter. Chemokine levels were determined using the BioPlex system from BioRad (Hercules, CA, USA) for the Luminex multi-analyte system. Chemokine levels were expressed as pg/mg synovium.

Statistical analysis

Differences between experimental groups were tested for significance using the Mann-Whitney U test. P values less than 0.05 were considered significant.

Results

IFN-γ-induced up-regulation of all three FcγRs in the synovial lining

As activating FcγRs expressed on synovial macrophages are important in the onset of ICA, the ability of IFN-γ to regulate FcγR expression in the syn-

Table 1 **FcγR mRNA levels in naive knee joints injected with AdeGFP or AdIFN-γ at different time points**

FcγR	Treatment	6 hr	Days 1	3	7
FcγRI	AdeGFP	1.6	0.5	0	0
	AdIFN-γ	4.0	6.2	6.5	3.7
FcγRII	AdeGFP	0.3	0.5	0	0
	AdIFN-γ	0.1	1.3	1.6	0.5
FcγRIII	AdeGFP	0.6	0.5	0	0
	AdIFN-γ	0.1	2.6	2.8	1.9

Expression profile of FcγRI, II, and III mRNA levels after injection of AdeGFP or AdIFN-γ in synovium samples isolated at different time points (6 hours, day 1, day 3, and day 7). Synovium samples from 4 knee joints were pooled in each experiment and mRNA was isolated. The Ct values of FcγRI, II, and III in naive knee joints were subtracted from the Ct values for FcγRs at different time points after injection. Ct values were corrected for GAPDH content for each individual sample. Data are the mean of 2 experiments.

ovium was investigated first. AdIFN-γ or the control AdeGFP virus was injected into naive knee joints of C57BL/6 mice and mRNA levels of the activating FcγRI and III and the inhibiting FcγRII were detected in synovial specimen. Injection of the control virus resulted in a slight increase of mRNA levels of FcγRI but not FcγRIII, which was back to baseline 3 days after injection (Table 1).

When AdIFN-γ was injected, a high level of IFN-γ was found in synovial washouts at day 1 (2870 pg/ml), which was already undetectable at day 2. This high peak of IFN-γ resulted in a significant increase of FcγRI mRNA already after 6 hours (ΔCt = 4), and this remained high until day 7 (ΔCt = 3.7) (Table 1). In contrast, FcγRIII mRNA levels were not yet elevated at 6 hours, but increased significantly thereafter. Moderate levels of FcγRIII were found both at 24 hours and 7 days after injection (ΔCt = 2.6 and ΔCt = 1.9 respectively), but these were evidently lower compared to levels of FcγRI. IFN-γ also induced up-regulation of inhibitory FcγRII mRNA at day 1 and 3 (respectively ΔCt=1.3 and ΔCt=1.6).

IFN-γ bypasses IC-mediated joint inflammation in FcγRIII$^{-/-}$ mice, resulting in inflammatory cell mass similar to that found in WT controls

In a previous study, we found that FcγRIII is the dominant activating receptor involved in the onset of ICA as cell influx was largely blocked in FcγRIII$^{-/}$

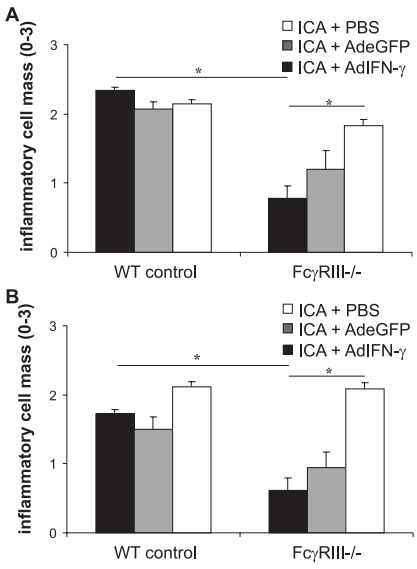

Figure 1
Joint inflammation in WT control and FcγRIII$^{-/-}$ arthritic knee joints determined as the amount of inflammatory cells in the synovium (A) and in the joint cavity (B) using an arbitrary scale from 0-3 (0, no; 1, minor; 2, moderate; 3, maximal). The inflammatory cell mass was significantly increased in FcγRIII$^{-/-}$ mice after injection with AdIFN-γ. Data are the mean ± SEM of 6 mice and significance was evaluated using the Mann-Whitney U test (*, P<0.05).

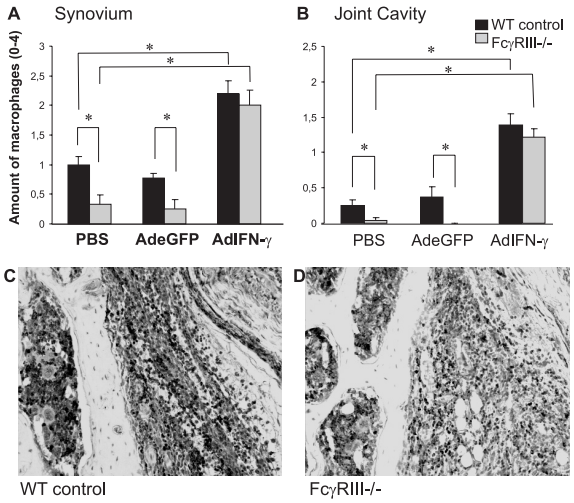

Figure 2
Macrophages in the synovial lining (A, infiltrate) and in the joint cavity (B, exudate) and myeloid-related protein 8 (MRP8) in WT controls (C) and FcγRIII$^{-/-}$ mice (D), 3 days after ICA induction. Macrophages were detected using an antibody against F4/80. Note that after injection of AdIFN-γ, the percentage of macrophages was comparable in WT controls and FcγRIII$^{-/-}$ mice, whereas injection of PBS or AdeGFP resulted in significantly less macrophages in FcγRIII$^{-/-}$ mice. Values represent the mean ± SEM of 6 mice. Representative sections showing MRP8 localization which was comparable in arthritic knee joints of WT control (C) and FcγRIII$^{-/-}$ (D) mice. (Original magnification x 200.) * = P<0.05, Mann-Whitney U test.

knee joints [11]. Injection of AdIFN-γ in FcγRIII$^{-/-}$ knee joints followed by ICA induction led to an elevation of 100% of the inflammatory cell mass at day 3, to a level comparable with that found in WT controls. In contrast, joint inflammation in PBS or control AdeGFP virus injected FcγRIII$^{-/-}$ arthritic knee joints remained significantly inhibited compared to WT controls (Figure 1). These results indicate that IFN-γ bypasses inhibition of joint inflammation.

In addition, we determined whether the inflammatory cell mass was of similar composition. Macrophages, the dominant cell type involved in cartilage destruction within this arthritis model, were detected using an antibody directed against F4/80. The activation state of the infiltrating inflammatory cells was determined using MRP8 and 14 as markers, which are associated with an activated phenotype of cells present in inflammatory sites. Using an arbitrary scale from 0-4, we found that the amount of macrophages in FcγRIII$^{-/-}$ arthritic knee joints injected with PBS or AdeGFP was low both in joint cavity and synovium compared with the amount of macrophages found in arthritic knee joints of WT mice. However, in IFN-γ-accelerated arthritis in FcγRIII$^{-/-}$ and WT control arthritic knee joints the percentage macrophages was similar (Figure 2A and B). Furthermore, it was

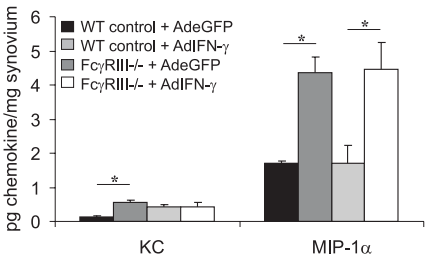

Figure 3
KC and MIP-1α levels measured in patella washouts from arthritic knee joints of WT controls and FcγRIII$^{-/-}$ mice injected with AdeGFP or AdIFN-γ. Note that IFN-γ induced a significant up-regulation of MIP-1α both in WT control and FcγRIII$^{-/-}$ mice. Values are expressed as mean ± SEM of 5 mice. * P<0.05, Mann-Whitney U-test.

found that the amount of MRP8 and 14 positive cells both in synovium and joint cavity was comparable in FcγRIII$^{-/-}$ mice and their WT controls after injection of AdIFN-γ (Figure 2C and D). MRP14 expression on cells in the synovial lining and in the joint cavity was identical to MRP8 expression (data not shown).

Complete restoration of chemokine production in FcγRIII$^{-/-}$ mice during IFN-γ-driven IC-mediated arthritis

In the presence of IFN-γ, the amount of inflammatory cells found in WT control and FcγRIII$^{-/-}$ arthritic knee joints was comparable. IFN-γ overexpression increased the influx of macrophages. Macrophage and neutrophil chemokine production in the arthritic knee joints was additionally investigated. MIP-1α (which is chemotactic for macrophages) and KC (which is chemotactic for PMNs) protein levels were determined in synovial washouts using the BioPlex method. Knee joints injected with AdIFN-γ showed a significant up-regulation of MIP-1α (from 1,5 to 4,5 pg/mg synovium), whereas levels of KC remained low (<1 pg/mg synovial tissue) which may explain the elevated macrophage influx (Figure 3). No significant differences were found between FcγRIII$^{-/-}$ and WT control synovial washouts (FcγRIII$^{-/-}$ and WT; 4.3 and 4.5 pg/mg synovial tissue, respectively).

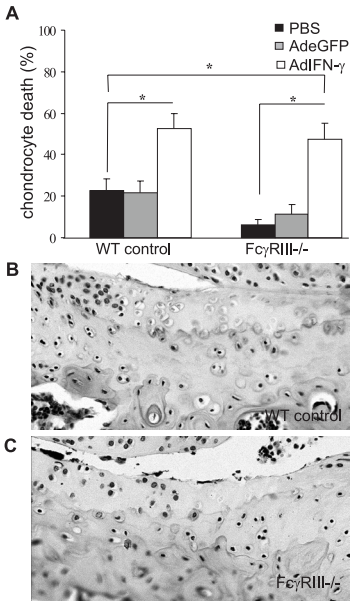

Figure 4
Chondrocyte death after injection of PBS, AdeGFP, or AdIFN-γ in WT controls and FcγRIII$^{-/-}$ mice (A), 3 days after arthritis onset. Note that chondrocyte death was significantly enhanced by IFN-γ both in FcγRIII$^{-/-}$ mice and WT controls. Bars show the mean ± SEM of 6 mice. Representative sections demonstrating chondrocyte death in cartilage layer of AdIFN-γ injected arthritic knee joints of WT controls (B) and FcγRIII$^{-/-}$ mice (C). (Original magnification x 400.) * = P<0.05, Mann-Whitney U test.

Lack of involvement of FcγRIII in regulating chondrocyte death in IFN-γ-stimulated IC-mediated arthritis

As in the presence of IFN-γ, the amount and activation state of macrophages in early-phase ICA is similar in FcγRIII$^{-/-}$ mice and WT controls, we further investigated whether IFN-γ also bypasses FcγRIII in late-phase cartilage destruction. Chondrocyte death is a characteristic feature in late-phase IC-mediated arthritis and is one of the causes of irreversible cartilage destruction. Chondrocyte death was determined in knee joints by measuring empty lacunae as a percentage of the total amount of chondrocytes in various cartilage layers. Injection of AdIFN-γ significantly elevated chondrocyte death (by up to 50%) in cartilage layers of WT controls and FcγRIII$^{-/-}$ mice (Figure 4).

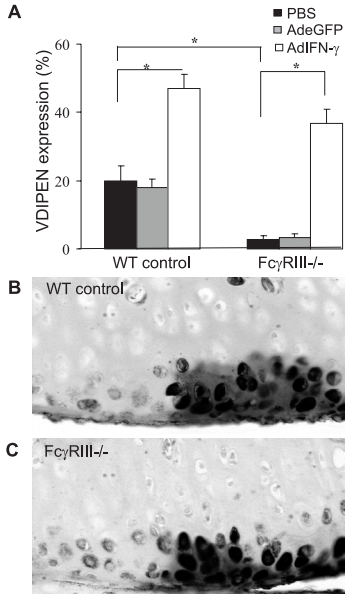

Figure 5
MMP-mediated proteoglycan damage (VDIPEN expression) after injection of PBS, AdeGFP, or AdIFN-γ in WT controls and FcγRIII$^{-/-}$ mice (A) 3 days after arthritis onset. Note that IFN-γ significantly elevated VDIPEN expression both in FcγRIII$^{-/-}$ and WT mice (A). Values represent the mean ± SEM of 6 mice. Representative sections demonstrating VDIPEN expression in AdIFN-γ injected arthritic knee joints of WT controls (B) and FcγRIII$^{-/-}$ mice (C). (Orginal magnification x 400.) * = P<0.05, Mann-Whitney U Test.

Enhancement of MMP-mediated cartilage destruction by IFN-γ in arthritic knee joints of FcγRIII$^{-/-}$ mice

In addition, we determined cartilage breakdown mediated by MMPs, which previously has been shown to be responsible for induction of severe irreversible breakdown of the cartilage matrix. MMP-mediated cartilage damage in arthritic knee joints was determined using immunolocalization of neoepitopes in proteoglycans (VDIPEN expression) and was scored in various cartilage layers within the knee joint.

Local overexpression of IFN-γ resulted in marked VDIPEN expression both in WT control and FcγRIII$^{-/-}$ knee joints (45% and 35%, respectively, in the total cartilage surface)(Figure 5) compared with knee joints that had received PBS and AdeGFP before onset of IC-mediated arthritis.

Discussion

In the present study, we demonstrated that the FcγRIII dependency of joint inflammation during ICA can be bypassed by local overexpression of IFN-γ. Furthermore, we showed that both activating FcγRI and III are able to initiate MMP-mediated cartilage damage, and we thereby confirmed the specific linkage between activation of FcγRI and chondrocyte death.

In a previous study using FcγRIII$^{-/-}$ mice, we found that the onset of ICA is highly FcγRIII-dependent, whereas ICA was not inhibited in FcγRI$^{-/-}$ mice [11]. Here we show that local IFN-γ expression in the knee joint can bypass this FcγRIII dependency. Synovial lining macrophages, which determine the onset of IC mediated arthritis [24-26], express low levels of FcγRIII whereas FcγRI is not expressed. IFN-γ induces strong up-regulation of activating FcγRI on macrophages and to a lesser extent, FcγRIII [27]. In accordance with this is our finding that local overexpression of IFN-γ in the knee joint significantly enhanced FcγRI expression in the synovium. These results led us to speculate that when FcγRI is highly expressed as in IFN-γ-stimulated ICA, joint inflammation could be induced by this receptor.

In the present study, the control virus also induced a slight increase in FcγRI expression. This is probably due to production of low amounts of IFN-γ by macrophages, as a response to the adenovirus [28]. This enhanced FcγRI expression can also account for the somewhat higher cell influx found in arthritic knee joints injected with AdeGFP compared with the PBS injected group.

T cells or T cell-derived cytokines are also able to regulate FcγR expression on macrophages either directly [29] or indirectly by producing cytokines like IFN-γ [30]. This can explain why during a T cell-mediated arthritis like antigen-induced arthritis (AIA), joint inflammation has been shown to follow a FcγRI-dependent pathway [12], whereas FcγRIII dependency is completely lost. In contrast, in non-T cell IC-mediated arthritis models, like the K/BxN model or our own passive IC model [11,31], joint inflammation was highly FcγRIII-dependent.

The increase in joint inflammation in FcγRIII$^{-/-}$ mice after onset of IFN-γ-stimulated IC arthritis was not due to a direct effect of IFN-γ, since overexpression of IFN-γ in naive knee joints induced a negligible amount of joint inflammation [17].

Since the percentage of macrophages is related to the severity of cartilage destruction [3,4], and no difference in inflammatory mass was present between WT control and FcγRIII$^{-/-}$ mice when IFN-γ was overexpressed, comparison of cartilage damage between these groups was simplified. MMPs mediate severe cartilage destruction found in IC-mediated arthritis. IL-1 induces chondrocytes to release latent MMPs that are stored in the cartilage matrix [32,33]. Moreover, synovial macrophages and fibroblasts are also involved in the production of latent MMPs [34]. Activation of pro-MMPs leads to destruction of proteoglycans and collagen type II fibers that form the cartilage matrix [13-15]. The factors involved in activation proMMPs are still not identified. However, recent studies using FcγR-deficient mice have shown that FcγRs are crucial in activation of latent MMPs [11,12].

Using FcγRIII$^{-/-}$ mice, we now demonstrated that FcγRI can mediate cartilage destruction by metalloproteinases. Up-regulation of FcγRI compensated absence of FcγRIII, resulting in comparable amounts of VDIPEN in cartilage layers of FcγRIII$^{-/-}$ mice and WT controls. Earlier, we found that IFN-γ overexpression in FcγRI$^{-/-}$ mice during ICA also enhanced VDIPEN expression [17]. Combining these results, it can be concluded that both FcγRI and FcγRIII have the potency to mediate MMP-mediated proteoglycan destruction.

Normally, the concentration of IFN-γ, which preferentially induces FcγRI expression and to a lesser extent FcγRIII, is low during experimental arthritis. However, during T cell dependent AIA, a shift of FcγRIII towards FcγRI was observed. FcγRI became the dominant receptor involved in MMP-cartilage damage, whereas FcγRIII dependency was completely lost [12]. In the present study, we found that the presence of high amounts of IFN-γ within the knee joint not only results in a shift in expression levels from FcγRIII to FcγRI, but also induces a strong up-regulation of FcγRIII. This may explain why FcγRIII still plays a role

in MMP-mediated cartilage destruction under these conditions.

Apart from differences in the amount and/or balance of the two activating FcγRs expressed within the synovium, there may also be a difference in the potency of the two receptors to drive severe cartilage destruction. In contrast to FcγRIII, FcγRI is a high affinity receptor for IgG. Binding of IgG-ICs results in production of oxygen radicals, which have been shown to be potent regulators of gene-activation through redox-signaling [35,36]. This may be reflected by chondrocyte death, another parameter for severe cartilage damage, which appeared to be significantly aggravated during IFN-γ-stimulated arthritis. Previously, we found that in FcγRI$^{-/-}$ mice, chondrocyte death remained low even in the presence of IFN-γ [17], indicating that FcγRI is the crucial FcγR receptor mediating this process. The specific role for FcγRI in chondrocyte death was confirmed in the present study, since IFN-γ overexpression in FcγRIII-/- mice resulted in high levels of chondrocyte death similar to those in controls. Since FcγRI is exclusively expressed on macrophages [37], this proves that macrophage activation is crucial in the induction of chondrocyte death.

Binding of IgG to FcγRI leads to intracellular signaling involving activation of phospholipase D-1 (PLD-1), and eventually leads to activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [38]. IFN-γ itself or products of FcγRI signaling might further augment NADPH-oxidase function. Elevation of the oxidative burst may lead to high concentrations of the relatively long-lived hydrogen peroxide (H_2O_2) [39]. H_2O_2 is able to act on more distant targets and easily penetrates through cell membranes and has been shown to kill cells by apoptosis [40]. In accordance with this, overproduction of the glycolytic enzyme glucose oxidase in the knee joint generated high levels of H_2O_2 and caused severe chondrocyte death [41].

Increased expression of FcγRI by IFN-γ is also found in RA patients. A previous study by Quayle et al. [42] reported that neutrophils isolated from synovial fluid of RA patients expressed higher levels of FcγRI, whereas no surface expression of FcγRI was detected on blood neutrophils from either patients or from healthy controls. This indicates that FcγRI expression is induced when inflammatory cells enter the diseased joint. Furthermore, it was found that stimulation of neutrophils from healthy controls with RA synovial fluid induced FcγRI expression and this stimulating effect could be abrogated by addition of anti-IFN-γ antibody [42]. This increase in FcγRI expression by IFN-γ may affect the ability to respond to IgG-containing ICs, which are abundantly present in synovial fluid and synovium from RA patients [5,6]. The present study underlines that enhanced FcγRI expression by IFN-γ in arthritic knee joints indeed alters the arthritic response, resulting in increased severity of cartilage destruction in experimental IC-mediated arthritis.

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Chapter 7

Local IL-13 gene transfer prior to immune complex-mediated arthritis inhibits chondrocyte death and matrix-metalloproteinase-mediated cartilage matrix degradation despite enhanced joint inflammation

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During immune complex-mediated arthritis (ICA) severe cartilage destruction is mediated by Fcγ receptors (FcγRs) (mainly FcγRI), cytokines (e.g.IL-1), and enzymes (matrix metalloproteinases (MMPs)). IL-13, a T helper 2 (Th2) cytokine abundantly found in synovial fluid of patients with rheumatoid arthritis, has been shown to reduce joint inflammation and bone destruction during experimental arthritis. However, the effect on severe cartilage destruction has not been studied in detail. We have now investigated the role of IL-13 in chondrocyte death and MMP-mediated cartilage damage during ICA. IL-13 was locally overexpressed in knee joints after injection of an adenovirus encoding IL-13 (AxCAhIL-13), one day before arthritis onset; injection of AxCANI (an empty adenoviral construct) was used as a control. IL-13 significantly increased the amount of inflammatory cells in the synovial lining and the joint cavity, by 30 to 60% at day 3 after ICA onset. Despite the enhanced inflammatory response, chondrocyte death was diminished by two-thirds at days 3 and 7. The mRNA level of FcγRI, a receptor shown to be crucial in the induction of chondrocyte death, was significantly downregulated in synovium. Furthermore, MMP-mediated cartilage damage, measured as neoepitope (VDIPEN) expression using immunolocalization, was halved. In contrast, mRNA levels of MMP-3, -9, -12 and –13 were significantly higher and IL-1 protein, which induces production of latent MMPs, was increased fivefold by IL-13. This study demonstrates that IL-13 overexpression during ICA diminished both chondrocyte death and MMP-mediated VDIPEN expression, even though joint inflammation was enhanced.

One of the main pathological features of rheumatoid arthritis is marked destruction of cartilage [1]. This destruction starts with reversible proteoglycan depletion, which is followed by irreversible cartilage degradation defined as chondrocyte death and breakdown of collagen type II eventually leading to matrix erosion. The latter is mainly induced by matrix metalloproteinases (MMPs), which generate specific cleavage sites within matrix molecules [2,3]. MMPs are secreted in an inactive form by IL-1-stimulated chondrocytes, synovial macrophages, and fibroblasts [4-6]. Activation of MMPs is still poorly understood, but MMP activity is primarily found in experimental immune complex (IC)-dependent arthritis models.

Immunoglobulin G (IgG)-containing ICs can activate macrophages upon recognition by Fcγ receptors (FcγRs). Three classes of murine FcγRs can be distinguished: FcγRI, II, and III. Triggering FcγRI and III activates cellular responses, whereas FcγRII is an inhibitory receptor [7]. Previous studies have showed that activating FcγRI and III are crucial in induction of severe cartilage destruction, since chondrocyte death and MMP-mediated cartilage damage were absent in FcγR-deficient mice after induction of immune complex-mediated arthritis (ICA) [8]. Furthermore, cartilage damage is aggravated by local overexpression of the proinflammatory T helper (Th)1 cytokine interferon (IFN)-γ [9]. This increase in

cartilage destruction was observed only in IC-dependent arthritis models [9]. FcγRI was found to be crucial in the induction of chondrocyte death, whereas both FcγRI and III mediated MMP-induced VDIPEN expression [9].

Since the Th1 cytokine IFN-γ worsens the arthritic response by up-regulation of the activating FcγRs, overexpression of a Th2 cytokine during arthritis might be protective, because of down-regulation of these receptors. In earlier studies, we found that adenoviral overexpression of IL-4 resulted in reduced MMP-mediated cartilage damage and chondrocyte death during ICA and arthritis induced by collagen type II [10,11]. IL-4 is regarded as a potent anti-inflammatory cytokine by direct inhibition of proinflammatory cytokines such as IFN-γ, IL-1, and tumour necrosis factor α [12]. However, IL-4 protein and mRNA are hardly detected in synovial fluid and synovium of rheumatoid arthritis patients [13]. In contrast, IL-13 is expressed in rheumatoid arthritis synovial fluid and synovial fluid macrophages and resembles many functions of IL-4 [14,15]. Systemic overexpression of IL-13 in collagen type II-induced arthritis and local overexpression of IL-13 in rat adjuvant-induced arthritis reduced joint inflammation and bone destruction [16,17]. However, the effect of IL-13 on cartilage destruction was not investigated in detail in these studies and remains to be elucidated.

In the present study we investigated whether IL-13 influences the development of chondrocyte death and MMP-mediated VDIPEN expression in ICA. Subsequently, regulation of FcγRs, MMP, and IL-1 expression by IL-13 was studied, as these are important mediators in severe cartilage damage.

The present study demonstrates that overexpression of IL-13 in arthritic knee joints, reduces chondrocyte death and MMP-mediated VDIPEN expression despite enhanced joint inflammation. Injection of an adenovirus encoding for IL-13 diminished chondrocyte death, which correlated with down-regulation of FcγRI expression in the synovium. Reduction of MMP-mediated VDIPEN expression was not reflected by MMP mRNA and IL-1 concentrations, as these were increased.

Material and Methods

Animals

C57Bl/6 male mice (10 to 12 weeks old) were purchased from Elevage-Janvier (Le Genest Saint Isle, France). Mice were fed a standard diet and tapwater ad libitum. Ethical approval was obtained from the research ethics committee of the Central Animal Facility in Nijmegen.

Local gene transfer of IL-13

The recombinant adenovirus encoding human IL-13 (AxCAhIL-13) was generated as described before [17-19] and an empty adenoviral construct (AxCANI) was used as control virus. AxCAhIL-13 or AxCANI (1.10^7 plaque-forming units) was injected intra-articularly in naive knee joints. Patellae with adjacent synovium were dissected in a standardized manner [20] and synovial biopsies were taken with a biopsy punch (diameter of 3 mm). Total RNA was extracted in 1 ml TRIzol reagent and used for quantitative PCR as described below. AxCAhIL-13

or AxCANI was injected intra-articularly one day before the induction of arthritis.

Induction of immune complex-mediated arthritis

Rabbit polyclonal antibodies directed against lysozyme were injected intravenously into mice. ICA was then passively induced by injecting 3 μg lysozyme coupled to poly-L-lysine in 6 μl pyrogen-free saline into the knee joints.

Histology of arthritic knee joints

Total knee joints were dissected at days 3 and 7 after the onset of arthritis. Joints were decalcified, dehydrated, and embedded in paraffin. Tissue sections (7 μm) were stained with hematoxylin and eosin. Histopathological changes were scored in two ways. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity. Chondrocyte death was scored as the amount of empty lacunae expressed as a percentage of the total number of cells within the cartilage layers.

Immunohistochemical detection of macrophages and polymorphonuclear neutrophils (PMNs)

Macrophages were detected using a specific antibody against F4/80, a murine macrophage membrane antigen [21]. PMNs were visualized using NIMPR14, a specific rat anti-mouse monoclonal antibody [22]. Primary antibodies were detected using rabbit anti-rat IgG and avidin-horseradish-peroxidase conjugate. Finally, sections were counterstained with hematoxylin. Macrophage and PMN subsets were quantitatively measured using an image analysis system. The inflammatory cell mass was selected by hand and the amount of positive features present in this area was displayed using a computer imaging system. Three sections of each knee joint were measured and the mean was calculated. We report the amount of positive features per 100,000 μm^2 inflammatory cell mass in the synovium.

Immunohistochemical VDIPEN staining

Sections were digested with proteinase-free chondroitinase ABC (0.25 units/ml in 0.1 M Tris HCl, pH 8.0 (Sigma, Zwijndrecht, The Netherlands)) to remove the side chains of proteoglycans followed by incubation with affinity-purified rabbit anti-VDIPEN IgG [23]. The primary antibody was detected using biotinylated goat anti-rabbit IgG, and avidin-streptravidin-peroxidase (Elite kit, Vector, Burlingame, CA, USA). Counterstaining was done with orange G (2%). Areas of immunostaining were expressed as a percentage of the total cartilage surface.

Quantitative detection of FcγR and MMP mRNA using RT-PCR

Specific mRNA-levels for FcγRI, II, and III and MMP-3, -9, -12, -13, and -14 were detected using the ABI/PRISM 7000 Sequence Detection System (ABI/PE, Foster City, CA). Briefly, 1 μg of synovial RNA was used for RT-PCR. mRNA was reverse transcribed to cDNA using oligodT primers. cDNA (1/100) was used in one PCR amplification. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C fol-

lowed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C with data collection in the last 30 seconds. Message for murine FcγRI, II, and III and MMP-3, -9, -12, -13, and –14 was amplified using the primers listed in Table 1 (Biolegio, Malden, The Netherlands) at a final concentration of 300 nM. Relative quantification of the PCR signals was performed by comparing the cycle threshold value (Ct) of the FcγR and MMP genes in the different samples after correction of the GAPDH content for each individual sample.

Table 1. Primers for detection of murine FcγR and MMP mRNA.

	Primer Sequence (5' - 3')
GAPDH	
Up	GGCAAATTCAACGGCACA
Low	GTTAGTGGGGTCTCGCTCCTG
FcγRI	
Up	ACACAATGGTTTATCAACGGAACA
Low	TGGCCTCTGGGATGCTATAACT
FcγRII	
Up	GACAGCCGTGCTAAATCTTGCT
Low	GTGTCACCGTGTCTTCCTTGAG
FcγRIII	
Up	GACAGGCAGAGTGCAGCTCTT
Low	TGTCTTCCTTGAGCACCTGGAT
MMP-3	
Up	TGGAGCTGATGCATAAGCCC
Low	TGAAGCCACCAACATCAGGA
MMP-9	
Up	GGAACTCACACGACATCTTCCA
Low	GAAACTCACACGCCAGAAGAATTT
MMP-12	
Up	GGACATGAAGCGTGAGGATGT
Low	GAAGTCTCCGTGAGCTCCAAAT
MMP-13	
Up	ACCTTGTGTTTGCAGAGCACTAACTT
Low	CTTCAGGATTCCCGCAAGAGT
MMP-14	
Up	AAGGCTGATTTGGCAACCAT
Low	GTCCCAAACTTATCCGGAACAC

Primer sequences used for RT-PCR on synovium.

Determination of cytokine and chemokine concentrations

To determine concentrations of IL-13, IL-1β, KC (a mouse homologue for human IL-8), and macrophage inflammatory protein 1α in patella washouts, synovial specimens were isolated in a standard manner [20] and incubated in 200 µl RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) for 1 hour at room temperature. Cytokine and chemokine concentrations were determined using the BioPlex system from BioRad (Hercules, CA, USA) for the Luminex multi-analyte system and expressed as pg/ml.

Statistical analysis

Differences between experimental groups were tested for significance using the Mann-Whitney U test. P values less than 0.05 were considered statistically significant.

Results

Local IL-13 expression in naive knee joints using adenoviral gene transfer

The expression of IL-13 was determined in synovial washouts at days 1, 2, 3, and 7 after injection of the AxCAhIL-13 virus. IL-13 reached a concentration of 0.4 ng/ml after 24 hours. Values increased to 2 ng/ml at day 2 and remained high up to 7 days after injection (Figure 1A). IL-13 was not detected after injection of AxCANI.

We next investigated whether injection of the adenoviral IL-13 construct causes joint inflammation by itself. Using histology, we found that IL-13 overexpres-

sion in naive knee joints did not recruit inflammatory cells at days 1, 2, 3, and 7 (Figure 1C). Injection of AxCANI resulted in minor cell influx in the synovial lining and joint cavity (Figure 1B), which was not detectable from day 2 onwards.

IL-13 overexpression during ICA enhances joint inflammation and alters the composition of the cell mass

To investigate whether IL-13 overexpression ameliorated the arthritic response, we injected AxCAhIL-13 one day before ICA induction. Joint inflammation was studied 3 and 7 days after arthritis onset.

IL-13 overexpression significantly increased the inflammatory cell mass in joint cavity and synovium, by 60% and 30%, respectively, 3 days after arthritis induction (Figure 2A). After 7 days, joint inflammation seemed to normalize in the IL-13 group (Figure 2B).

To further investigate inflammatory cell types attracted by IL-13, PMNs and macrophages were detected using specific NIMPR14 and F4/80 antibodies respectively using immunolocalization. At day 3, the amount of PMNs and macrophages was not markedly altered by IL-13 (Figure 3A and B). At day 7, however, the amount of PMNs in the synovial lining was 10 times higher (Figure 3A), whereas the amount of macrophages in the IL-13 group was half that in

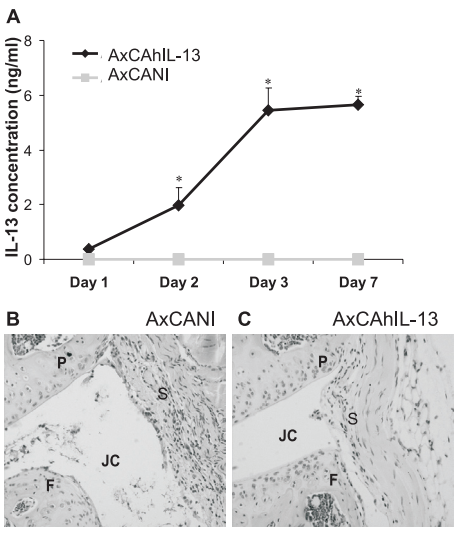

Figure 1
Adenoviral vector-mediated IL-13 expression in knee joints of C57Bl/6 mice. (A) Naive knee joints and (B) total knee joint sections 24 hours after injection of AxCANI (adenovirus encoding nog gene) or (C) AxCAhIL-13 (adenovirus encoding IL-13). Injection of AxCAhIL-13 resulted in 0.4 ng/ml IL-13 at day 1, which increased to 5.5 ng/ml by day 7 (A). Injection of AxCANI resulted in a mild thickening of the synovial lining (S), and some invading inflammatory cells in the joint cavity (JC) (B), whereas no inflammation was observed after AxCAhIL-13 injection (C). Plotted values are means ± SEM of data from 5 mice. * P < 0.05. Original magnification 200X. F, femur ; P, patella.

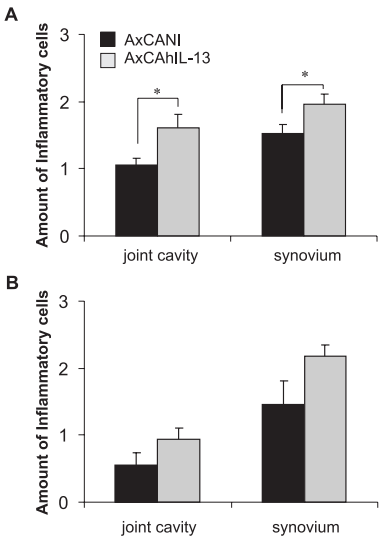

Figure 2
Joint inflammation in arthritic knee joints of C57Bl/6 mice injected with AxCANI (adenovirus encoding no gene) or AxCAhIL-13 (adenovirus encoding IL-13). At (A) day 3 and (B) day 7 after the onset of immune complex-mediated arthritis. The inflammatory cell mass was significantly enhanced by IL-13, in both the joint cavity and the synovium 3 days after arthritis induction. Bars show the means ± SEM for 10 mice. Significance was evaluated using the Mann-Whitney U test. *, P<0.05.

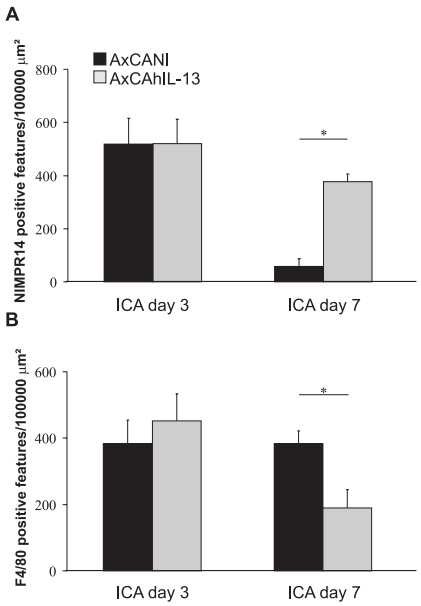

Figure 3
Immunohistochemical detection of inflammatory cells in knee joints of mice with immune complex-mediated arthritis (ICA). (A) Polymorphonuclear neutrophils (PMNs) and (B) macrophages in synovium 3 and 7 days after injection of AxCANI or AxCAhIL-13. PMNs were detected using the specific rat anti-mouse monoclonal antibody NIMPR14, and macrophages were detected using an antibody against the membrane marker F4/80. At day 7, the amount of NIMPR14-positive features was significantly higher in the synovium of AxCAhIL-13 injected arthritic knee joints, while the amount of F4/80 positive features was significantly lower. The bars represent means ± SEM for 10 mice. Data were evaluated using the Mann-Whitney U test. *, P<0.05.

the mice without IL-13 (Figure 3B).

KC concentration in synovial washouts is augmented by IL-13

A possible mechanism by which IL-13 can increase joint inflammation in the presence of ICs is elevation of chemokine production. To investigate this, synovial washouts were done on days 3 and 7, and the chemokines KC (chemotactic for neutrophils) and macrophage inflammatory protein 1α (chemotactic for macrophages) were measured. Local IL-13 overexpression increased KC concentrations 4- and 18-fold, respectively, at days 3 and 7 after arthritis induction, which correlates with the high amount of PMNs (Table 2). Macrophage inflammatory protein 1α concentrations at day 3 were comparable between the control and IL-13 groups. At day 7, macrophage inflammatory protein 1α expression was slightly increased by IL-13 (Table 2).

IL-13 strongly inhibits chondrocyte death during ICA: down-regulation of FcγRI

Because IL-13 enhanced the inflammatory response, we next investigated the effect of IL-13 overexpression on cartilage destruction. A characteristic feature of irreversible cartilage damage is chondrocyte death; this was scored as the percentage of empty lacunae relative to the total amount of chondrocytes present in various cartilage layers in the knee joint.

Three days after ICA induction, chondrocyte death, expressed as the mean for 6 cartilage layers in the knee joint, was very low in the IL-13 group (5%) and significantly less than in the control

Table 2 **Effect of IL-13 on chemokine concentrations (pg/ml) in arthritic knee joints in mice**

	KC	MIP-1α
ICA day 3		
AxCANI	56 ±8	303 ±6.8
AxCAhIL-13	196 ±31*	344 ±96
ICA day 7		
AxCANI	10 ±6	157 ±25
AxCAhIL-13	184 ±26*	268 ±98

Concentrations of KC and MIP-1α were detected in synovial washouts of arthritic knee joints 3 and 7 days after arthritis induction. KC concentrations were significantly higher in arthritic knee joints injected with AxCAhIL-13 both at day 3 and 7. *, P<0.05 in comparison with AxCANI.

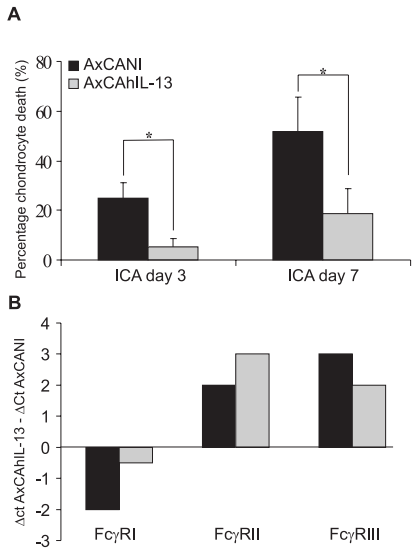

Figure 4

Chondrocyte death in the knee joints of mice with immune complex-mediated arthritis (ICA). (A) At day 3 and 7 in arthritic knee joints injected with AxCANI or AxCAhIL-13 and (B) expression profiles of Fcγ receptor I (FcγRI), II, and III mRNA levels induced by IL-13 in synovium. IL-13 significantly decreased chondrocyte death, both at day 3 and at day 7 (A). Cycle threshold (Ct) values of FcγRI, II, and III in arthritic knee joints injected with AxCANI were subtracted from the Ct values for FcγRs after injection of AxCAhIL-13. Ct values were corrected for GAPDH content for each individual sample. (B) FcγRI mRNA level was down-regulated by IL-13, whereas an up-regulation was observed for both FcγRII and III (B). Bars represent means ± SEM for 10 mice. Mann-Whitney U test. * $P<0.05$.

arthritic knee joints, which showed 25% chondrocyte death (Figure 4A). At day 7, chondrocyte death was even more significantly reduced (65%) in comparison with the control group (Figure 4A).

In a previous study, we found that FcγRI is the dominant receptor mediating chondrocyte death during ICA [9]. We speculated that the decreased chondrocyte death might be caused by down-regulation of FcγRI by IL-13. For that reason, we determined the effect of IL-13 on mRNA levels of all three classes of FcγRs in synovium. Cycle values of FcγRI, II, and III in synovium of arthritic knee joints injected with AxCANI were subtracted from cycle values of FcγRs after AxCAhIL-13 injection. Interestingly, FcγRI mRNA level was decreased by IL-13 at day 3 after ICA induction ($\Delta Ct = 2$), and was still slightly down-regulated at day 7 ($\Delta Ct = 0,5$). In contrast, FcγRII and FcγRIII were up-regulated by IL-13, at both days 3 and 7 after ICA induction (Figure 4B).

IL-13 increases IL-1 production and MMP mRNA levels in the arthritic knee joint

Cartilage matrix degradation is largely mediated by MMPs. Production of latent MMPs is mainly regulated by IL-1 and this cytokine has been shown to be crucial in the generation of MMP-mediated neoepitopes [23]. The production of IL-1 was determined in synovial washouts of arthritic knee joints at both days 3 and 7. At day 3, IL-1 concentration was between 450 and 500 pg/ml in both the control and the IL-13 group. However, at day 7, the IL-1 level was reduced in the control group but remained high in the IL-13 group (control; 54 pg/ml vs IL-13; 255 pg/ml).

This sustained IL-1 production at day 7 may result in high concentrations of MMPs in synovium. Levels of MMP-3, -9, -12, -13, and -14 mRNA were detected by quantitative PCR. MMP-12 mRNA levels were increased 10-fold and 8-fold by IL-13 at days 3 and 7, respectively, after the onset of ICA. At day 7, mRNA levels of MMP-3, -9, and -13 were also significantly increased in the IL-13 group (Table 3).

Table 3 Effect of IL-13 on MMP mRNA levels in synovium of mice with ICA

	ICA day 3		ICA day 7	
	AxCANI	AxCAhIL-13	AxCANI	AxCAhIL-13
MMP-3	5.7±0.3	7.1±0.8	4.2±0.5	6.1±0.2*
MMP-9	5.1±0.2	4.8±0.3	0.2±0.6	3.9±0.5*
MMP-12	0.6±0.4	5.8±1.1*	0.9±1	8.1±0.9*
MMP-13	3.2±0.2	2.7±0.3	4.3±0.3	6.4±0.3 *
MMP-14	3.7±0.4	4.9±0.8	3.7±1	3.7±0.6

Expression profile of MMP-3, -9, -12, -13, and –14 mRNA levels after injection of AxCANI or AxCAhIL-13 in synovial biopsies isolated at day 3 and day 7 after arthritis onset. The Ct values for MMP genes in naive knee joints were subtracted from the Ct values for MMPs at day 3 and 7 after arthritis onset. Ct values were corrected for GAPDH content for each individual sample. Note that MMP-3, -9, -12, and –13 mRNA levels were significantly increased at day 7 by IL-13, and the MMP-12 mRNA level was already elevated at day 3. Values represent the means ± SEM for 5 mice. * P<0.05, Mann-Whitney U-test.

MMP-mediated VDIPEN expression is reduced by IL-13 overexpression

Increased IL-1 and MMP concentrations may induce enhanced MMP-mediated proteoglycan degradation and this was further investigated by detection of VDIPEN neoepitope expression in the cartilage. In the control group, 35% of the cartilage surface expressed VDIPEN neoepitopes after 3 days (Figure 5). Injection with AxCAhIL13 reduced VDIPEN expression by 43%, as only 20% VDIPEN expression was found in the IL-13 group. The inhibitory effect of IL-13 was still present at day 7 after arthritis induction, as only 10% VDIPEN expression was found in the IL-13 group compared to 25% in the control group (Figure 5).

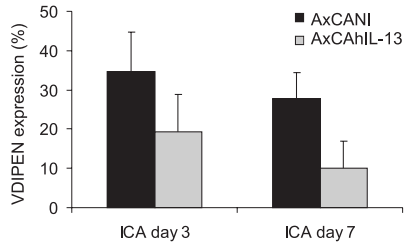

Figure 5
Matrix metalloproteinase-mediated aggrecan damage in knee joints of mice with immune complex-mediated arthritis. VDIPEN expression at day 3 and 7 after ICA induction in knee joints injected with AxCANI or AxCAhIL-13. Note that VDIPEN expression was reduced by IL-13 both at day 3 and day 7. Values represent the mean ± SEM for 10 mice. * P<0.05, Mann-Whitney U test.

Discussion

In the present study, we have shown that local gene transfer of IL-13 reduced severe cartilage destruction defined as chondrocyte death and MMP-mediated aggrecan damage during ICA.

Local IL-13 overexpression during IC-dependent arthritis enhanced joint inflammation. To exclude the possibility that IL-13 itself induces influx of inflammatory cells, as is found when IL-13 is overexpressed in the lung [24,25], AxCAhIL-13 was injected in naive knee joints. We observed that IL-13 overexpression in the knee joint did not recruit inflammatory cells. This observation indicates that overexpression of IL-13 induces elevated joint inflammation in combination with IC triggering. In our IC-dependent arthritis model, we showed that joint inflammation is determined by

activating FcγRIII [26]. In the present study, we find that IL-13 increased expression of FcγRIII within the synovium, which is not in line with the study showing that IL-13 decreases FcγRIII expression on human monocytes [27]. However, regulation of FcγR expression on mouse macrophages by IL-13 has not been described. IL-13 has high similarity with IL-4, which can increase FcγRIII expression on murine mast cells [28]. Binding of IC to FcγRIII on macrophage lining cells leads to activation, resulting in elevated influx of inflammatory cells.

We further found that overexpression of IL-13 in arthritic knee joints particularly increased the amount of PMNs. This is in line with earlier studies in which it was shown that stimulation of FcγRIII induces release of PMN attracting chemokines as IL-8, resulting in neutrophil accumulation [29-31]. The proinflammatory action of IL-13 found in the present study seems to be dependent on costimulation with ICs to trigger arthritis onset, since local overexpression of IL-13 during T cell-mediated rat adjuvant-induced arthritis diminishes joint inflammation [17]. In the latter model, ICs do not play a role.

Whether IL-13 decreases or enhances joint inflammation may also be dependent on systemic or local overexpression. Systemic overexpression of IL-13 during collagen type II-induced arthritis, in which FcγRIII is also required for arthritis development [32], decreased joint inflammation [16]. An explanation may be that systemic overexpression of IL-13 hampers the development of the immune response by induction of isotype switching to the nonarthritogenic IgG4 and IgE [33,34], thereby ameliorating the arthritic response. Induction of immunity is hardly affected by local overexpression, as was shown when injection of AdIL-4 (adenovirus encoding IL-4) in knee joints during arthritis induced by collagen type II markedly increased the amount of inflammatory cells [11].

Cartilage destruction during ICA is mostly related to joint inflammation. Despite the enhanced influx of inflammatory cells, however, a significant reduction of chondrocyte death was induced by IL-13. Chondrocyte death may be the result of increased production of oxygen radicals, as reactive oxygen species can mediate apoptosis [35]. In a previous study, we showed that there is a prominent role for FcγRI mediating chondrocyte death during ICA. In FcγRI-deficient mice, chondrocyte death was almost absent. When the Th1 cytokine IFN-γ was overexpressed, a significant increase in chondrocyte death was observed, which was dependent on FcγRI [9]. Stimulation of FcγRI leads to production of oxygen radicals via NADPH-oxidase [36]. In the present study, we find that in knee joints injected with AxCAhIL-13, FcγRI expression remained low, whereas in knee joints injected with control virus, FcγRI expression level was enhanced in the synovium. The decrease in chondrocyte death might be due to a reduced FcγRI concentration. Moreover, it has been shown that IL-13 itself down-regulates production of oxygen radicals by inflammatory cells, since IL-13 can inhibit protein kinase C-triggered respiratory burst in monocytes [37]. The inhibiting effect of IL-13 on oxygen radical production seemed to be monocyte-dependent, as no reduction was found in PMNs [38].

In addition, IL-13 also reduced MMP-mediated VDIPEN neoepitope expression. It has been reported that IL-13 diminishes the breakdown of collagen and proteoglycans from bovine cartilage, by regulation of MMP expression [39]. Several mechanisms may inhibit MMP-mediated cartilage destruction, as regulation of MMPs occurs at three different levels: MMP synthesis, activation of latent enzyme, and MMP-inhibition. IL-1 is a prominent cytokine controlling the production of latent MMPs [40] and diminished production of IL-1 might reduce MMP-mediated cartilage damage. We found, however, that IL-13 overexpression in arthritic knee joints strongly increased IL-1β concentrations. IL-13 is described as an anti-inflammatory cytokine, which in general reduces IL-1β production [14,27,41]. However, the effect of IL-13 on IL-1 production by IC-stimulated macrophages has not been described to date. In addition to macrophages, fibroblasts and PMNs are also present in the knee joint at day 7 after the onset of arthritis.

The sustained production of IL-1 by IL-13 may indeed stimulate MMP production, as reflected by enhanced MMP-3, -9, -12, and -13 mRNA levels 7 days after ICA induction in AxCAhIL-13-injected arthritic knee joints. MMP-12 mRNA level was already increased at day 3 after the onset of arthritis. It has been shown that MMP-12 expression is IL-13 dependent and that MMP-12 is a critical downstream mediator and regulator of IL-13-induced responses [42,43]. Furthermore, IL-13 induction of MMP-2, -9, and -13 is at least partly mediated by MMP-12 [43], indicating that MMP-12 might be a crucial enzyme inducing MMP-mediated cartilage damage.

Furthermore, IL-13 might interfere at the level of activation of MMPs. MMPs are secreted in a latent form and activation occurs after cleavage of a propeptide. Factors that activate latent MMPs are still unknown. However, MMP-mediated VDIPEN expression is mainly found in IC-dependent arthritis models, in which FcγRs are of utmost importance. Down-regulation of the activating FcγRs might reduce VDIPEN expression. Indeed, we found that IL-13 strongly diminished FcγRI expression in synovium. Another mechanism involved in activation of MMPs, is production of oxygen radicals. As mentioned above, stimulation of FcγRI results in assembly of the NADPH-oxidase complex, which produces oxygen radicals [36]. Additionally, oxygen metabolites can be converted into H_2O_2, which can activate latent pro-MMPs [44,45]. Taken together, decreased FcγRI expression reduces the production of oxygen radicals, which apart from chondrocyte protection may also result in diminished MMP-mediated VDIPEN expression.

The present study shows that IL-13 is a potent cytokine that protects the cartilage matrix against degradation during ICA. In addition, these results indicate that regulation of the expression of FcγR, particularly FcγRI, might be involved in this process. Therefore, modulation of FcγRI by Th2 cytokines seems to be a promising therapeutic tool diminishing cartilage damage in rheumatoid arthritis.

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Chapter 8

NADPH-oxidase driven oxygen radical production determines chondrocyte death and partly regulates metalloproteinase-mediated cartilage matrix degradation during interferon-γ-stimulated immune complex-mediated arthritis

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In previous studies we found that FcγRI determines chondrocyte death and matrix metalloproteinase (MMP)-mediated cartilage destruction during IFN-γ-regulated immune complex-mediated arthritis (ICA). Binding of immune complexes (IC) to FcγRI leads to prominent oxygen radical production. In the present study, we now investigated the contribution of NADPH-oxidase driven oxygen radical production to cartilage destruction using P47phox$^{-/-}$ mice lacking a functional NADPH-oxidase complex. Induction of a passive IC-mediated arthritis in knee joints of P47phox$^{-/-}$ resulted in significant elevation of joint inflammation at day 3 when compared with wild-type (WT) controls as studied by histology. However, when IFN-γ was overexpressed by injection of adenoviral-IFN-γ in the knee joint before ICA induction, the inflammatory cell mass was comparable at both days 3 and 7, which comprised mainly macrophages in both mouse strains. Proteoglycan depletion in the cartilage layers of the knee joints was similar at days 3 and 7 in both groups. Aggrecan breakdown in cartilage caused by MMPs was further studied by immunolocalisation of MMP-mediated neoepitopes (VDIPEN). VDIPEN expression was significantly lower (between 30-60%) in IFN-γ stimulated P47phox$^{-/-}$ at day 7. This despite the strong up-regulation of mRNA levels of various MMPs such as MMP-3, -9, -12, and -13 in synovia of P47phox$^{-/-}$, as measured with quantitative RT-PCR . The latter suggests that oxygen radicals are involved in activation of latent MMPs. Chondrocyte death, determined as percentage of empty lacunae in articular cartilage, varied between 20-60% at day 3 and 30-80% at day 7 in WT mice, but was completely blocked in P47phox$^{-/-}$ at both time-points. FcγRI mRNA expression was significantly lower, whereas FcγRII and FcγRIII were higher in P47phox$^{-/-}$ when compared with controls. NADPH-oxidase driven oxygen radical production determines chondrocyte death and aggravates MMP-mediated cartilage destruction during IFN-γ-stimulated IC-mediated arthritis. Up-regulation of FcγRI by oxygen radicals may contribute to cartilage destruction.

During rheumatoid arthritis (RA), large amounts of inflammatory cells, mainly macrophages, migrate into the synovial layer [1]. Many of these macrophages become activated by as yet unknown mechanisms. Activated macrophages produce cytokines such as TNF-α and IL-1 and enzymes like matrix metalloproteinases (MMPs), which can mediate severe cartilage destruction. A strong correlation was found between the number of activated macrophages and cartilage erosion [2]. Important triggers of macrophage activation are IgG-containing immune complexes (ICs) which are found in large amounts in joints of many RA patients [3]. In previous studies, we have found that severe cartilage destruction mainly developed when ICs were present [4]. Severe cartilage destruction is thereby defined as chondrocyte death and cartilage matrix destruction. The latter is induced predominantly by MMPs, which are re-

leased in a latent form. Upon activation these enzymes degrade the collagen type II network in the cartilage, resulting in irreversible erosion [5]. During IC-mediated arthritides, synovial macrophages appeared to be dominant players in the induction of severe cartilage destruction [6].

IgG-containing ICs communicate with macrophages using Fcγ receptors (FcγR). Three classes have been described in the mouse and previous studies in our lab showed that absence of the activating FcγRI and III completely abrogated severe cartilage destruction [7-9].

The mechanism by which FcγR mediate chondrocyte death and MMP-mediated cartilage destruction is not known. However, recently we have found that FcγRI is the dominant activating FcγR causing cartilage destruction [10-11]. In T cell-driven IC-mediated arthritis, chondrocyte death in FcγRI$^{-/-}$ was completely abrogated, whereas MMP-mediated cartilage destruction was significantly diminished [12]. Moreover, IC-mediated arthritis stimulated by local overexpression of the T cell factor IFN-γ showed pronounced chondrocyte death, which was also completely mediated by FcγRI [13].

Binding of IC to FcγRI causes intracellular signaling and triggers activation of the multicomponent enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, which catalyzes the production of oxygen species [14]. The latter have been shown to be involved in cell death [15-16] and activation of MMPs [17]. The transmembrane cytochrome b558 plays a active central role in the NADPH-oxidase complex and it is comprised of two subunits: gp91phox and p22phox. P47phox is the cytosolic component of the NADPH-oxidase complex that translocates to the membrane and associates with cytochrome b556 to form the active complex that catalyzes the reduction of oxygen to superoxide. Functionally p47phox increases the binding of P67phox to cytochrome b556 by approximately 100 fold [18-20]. IFN-γ strongly stimulates p91 but also expression of FcγRI. Binding of ICs to FcγRI further increases NADPH-oxidase activity [21]. Phospholipase D-1 (PLD-1) has been shown to be an important mediator between FcγRI signaling and activation of NADPH-oxidase [14,22]. The combination of IFN-γ and FcγRI stimulation may therefore result in a strong stimulation of NADPH-oxidase catalyzing large amounts of superoxide production.

In the present study, we investigated the effect of NADPH-oxidase driven oxygen radicals in the generation of severe cartilage destruction during IFN-γ-accelerated IC-mediated arthritis. For that purpose p47phox gene-deficient mice were used which are unable to form a functional NADPH-oxidase complex [23] and therefore are unable to produce oxygen species by the NADPH-oxidase pathway. Other oxygen radical producing pathways however, remain intact. We find that chondrocyte death was completely abrogated, whereas MMP-mediated cartilage destruction was significantly inhibited. FcγRI expression was significantly downregulated, whereas on the contrary, MMP gene expression in the synovium was higher suggesting that oxygen radicals are involved in the activation step of MMPs.

Material and Methods

Animals

Mice deficient for the NADPH-oxidase complex (p47phox$^{-/-}$) generated as described previously [23], lack the cytosolic p47phox subunit of the NADPH-oxidase multicomponent system. The knock-out (KO) mice were backcrossed to the C57BL6 background for 15 generations and C57BL/6 (Jackson Laboratory, Bar Harbour,ME) were used as wild-type (WT) controls. In some experiments p47phox$^{-/-}$ mice of intercross progeny (C57BL/6 X 129Sv) were used with their proper controls. Colonies were maintained at the National Institutes of Health (Bethesda,MD). All mice were housed under specified pathogen-free conditions during breeding and experiments. Mice received autoclaved chow and acidified water ad libitum. Only mice that were healthy were used in the experiments and were age-matched (10 to 20 weeks) and sex matched for each set of experiments. All experiments were approved by local authorities of Animal Care and Use Committee.

In vivo overexpression of IFN-γ using an adenoviral construct

The recombinant adenovirus encoding murine IFN-γ (AdIFN-γ) was generated as described before [24]. Knee joints of naive mice were intra-articularly injected with 6 μl of AdIFN-γ (1.10^7 pfu). At different time points (day 3 and 7), patellae with adjacent synovium were dissected in a standardized manner [25] and synovium biopsies were taken using a biopsy punch with a diameter of 3 mm. Total RNA was extracted in 1 ml TRIzol reagent and used for quantitative PCR as described below. AdIFN-γ was intra-articularly injected one day before arthritis induction.

Induction of immune complex-mediated arthritis

Immune complex-mediated arthritis (ICA) was passively induced by injecting 3 μg poly-L-lysine coupled lysozyme in the knee joints of mice that had previously (16 hours earlier) received, intravenously, polyclonal antibodies directed against lysozyme. These antibodies were raised in rabbits.

Histology of arthritic knee joints

Total knee joints of mice were isolated 3 and 7 days after arthritis onset. Knee joints were decalcified, dehydrated, and embedded in paraffin. Tissue sections (7 μm) were stained with hematoxylin and eosin. Histopathological changes were scored using the following parameters. Inflammation was graded on a scale from 0 (no inflammation) to 3 (severe inflamed joint) as influx of inflammatory cells in synovium and joint cavity.

To study proteoglycan depletion from cartilage matrix, sections were stained with safranin O followed by counterstaining with fast green. Proteoglycan depletion (loss of red staining) from various cartilage layers was determined using an arbitrary scale from 0-3. Normal cartilage was taken as 0 value, whereas cartilage fully depleted of proteoglycans was taken as maximal 3 value. Chondrocyte death was determined in total knee joint sections stained with hematoxylin and eosin and expressed as

percentage of the area of the cartilage containing empty lacunae in relation to the total area. All experiments were scored separately and independently from each other.

Immunohistochemical detection of the identification marker of macrophages

F4/80, a murine macrophage membrane antigen, was detected using a specific rat anti-mouse F4/80 antibody. Primary antibodies were detected using rabbit anti-rat IgG and avidin-horseradish peroxidase conjugate. Finally, sections were counterstained with Mayer's hematoxylin (Merck, Germany).

Immunolocalization of MMP-induced neoepitope (VDIPEN)

For immunohistochemical analysis of MMP-induced neoepitopes, sections were deparaffinized, rehydrated and digested with chondroitinase ABC (Sigma, 0.25 U/ml, 0.1 M Tris-HCL pH 8.0) for 1 hour at 37°C to remove chondroitine sulphate from the proteoglycans. Sections were then treated with 1% H_2O_2 in methanol for 20 minutes and subsequently 5 minutes with 0.1% (v/v) triton X-100 in PBS. After incubation with 1.5% (v/v) normal goat serum for 20 minutes, sections were incubated with affinity-purified anti-VDIPEN IgG overnight at 4°C. These antibodies were kindly provided by Irwin Singer and Ellen Bayne (Merck Research Laboratories, Rahway, USA) and have been extensively characterized before [26, 27]. In addition, sections were incubated with biotinylated goat anti-rabbit IgG and binding detected using avidine-peroxidase staining (Elite kit, Vector Labs, Inc., Burlingham). Induction of the peroxidase product was detected using nickel enhancement and counterstaining was performed with orange G (2%) for 5 minutes.

Quantitative RT-PCR

RNA was isolated using 1 ml of TRIzol reagent (Life Technologies, Breda, The Netherlands). Specific mRNA levels for various MMPs (MMP- 2, -3, -9,-12, and -13), their inhibitors (TIMP-1, -2, -3, and -4) and FcγR (FcγRI, II, and III) were quantified using the ABI/PRISM 7000 Sequence Detection System (ABI/PE, Foster City, CA). Briefly, 1 μg of synovial RNA was used for reverse transcriptase-PCR. mRNA was reverse-transcribed to cDNA using oligodT primers and 1/100 of the cDNA was used in one PCR amplification. PCR was performed in SYBR Green Master Mix using the following amplification protocol: 2 minutes at 50°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C with data collection in the last 30 seconds. Message for murine GAPDH, MMPs, MMP inhibitors and FcγR was amplified using specific primers (Biolegio, Malden, The Netherlands) for these molecules at a final concentration of 300 nmol/L. Relative quantification of the PCR signals was performed by comparing the cycle threshold (Ct) value of the various molecules in the different samples after correction of the GAPDH content for each individual sample to rule out confounding by variation of the RNA purification and reverse transcriptase step.

Results

During IC-mediated arthritis joint inflammation is downregulated by oxygen radicals, which is compensated by IFN-γ

To investigate the effect of NADPH-oxidase driven oxygen radical production on joint inflammation, ICA was induced in knee joints of P47phox$^{-/-}$ and their wild-type (WT) controls. Total knee joint sections were stained with hematoxylin/eosin and the amount of inflammatory cells present within the synovium (infiltrate) and joint cavity (exudate) were determined using an abitrary scale ranging from 0-3. At day 3 after induction of ICA, joint inflammation was significantly higher in P47phox$^{-/-}$ when compared with WT controls (Figure 1), indicating that oxygen radicals inhibit IC-mediated joint inflammation.

In addition, the effect of IFN-γ on joint inflammation was investigated by injecting an adenoviral construct encoding IFN-γ into knee joints of P47phox$^{-/-}$ and their WT controls, one day before ICA induction. Earlier studies have shown that IFN-γ does not elevate joint inflammation during ICA, whereas cartilage destruction is significantly enhanced [13]. The latter is strongly correlated to upregulation of FcγRI. We found that the amount of inflammatory cell mass was comparable in IFN-γ-stimulated knee joints of P47Phox$^{-/-}$ and their WT controls, both at days 3 and 7 after ICA induction (Figure 2A-C).

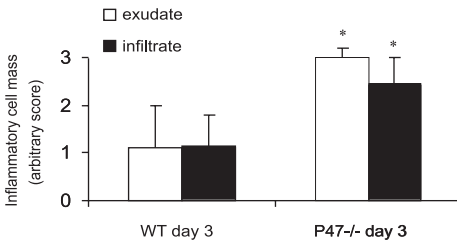

Figure 1
Joint inflammation 3 days after induction of ICA in P47phox$^{-/-}$ and their WT controls. The amount of cells present in the synovium (infiltrate) and in the joint cavity (exudate) was determined using an arbitrary scale from 0-3 (0, no cells; 1, minor; 2, moderate; 3, maximal). The amount of cells was determined blindly by two independent observers. Data are the mean ± SD of 8 animals. Significance was tested using the Wilcoxon rank test (*, P<0.05).

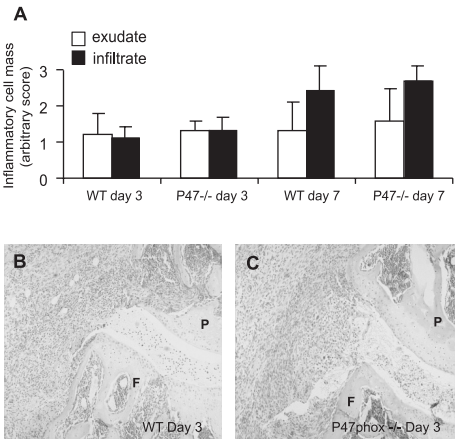

Figure 2
Joint inflammation 3 and 7 days after induction of IFN-γ-stimulated ICA in P47phox$^{-/-}$ and their WT controls. The amount of cells present in the synovium (infiltrate) and in the joint cavity (exudate) was determined using an arbitrary scale from 0-3 (see Figure 1 for definition). Note that there is a comparable cell mass in arthritic knee joints of P47phox$^{-/-}$ and WT controls. (A and C versus WT control B). Significance was tested using the Wilcoxon rank test (*,P<0.05). Original magnifications, x 100. F,femur ; P=patella.

Apart from the amount, also the cell type of the infiltrated cells may be different. To determine the contribution of the macrophage, which is the dominant cell type involved in cartilage destruction within this model, sections were stained with antibodies directed against F4/80. At day 7 after IFN-γ-stimulated ICA induction, high but comparable amounts of F4/80-positive cells were detected in

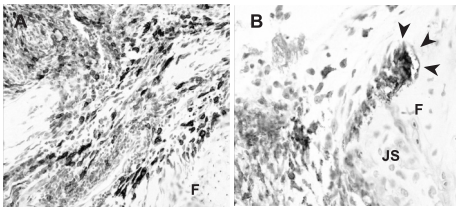

Figure 3
Expression of the macrophage marker F4/80 7 days after induction of IFN-γ-stimulated ICA. Note that 70-80% of the infiltrated cells within the synovium consist of macrophages (A). F4/80 positive macrophages attached to the cartilage surface and were found in the lacunae of erosion pits (B, see arrows). Original magnification, x 400. F,femur; JS,joint space.

both P47phox$^{-/-}$ mice and their WT controls. Between 70-80% of the inflammatory cells, both in infiltrate and exudate showed clear F4/80 staining (Figure 3A). Moreover, large amounts of F4/80 positive macrophages attached to cartilage surfaces at sites where erosion was detected (Figure 3B).

Oxygen radicals are not involved in mediating early proteoglycan depletion

During ICA, mild cartilage destruction starts with the release of proteoglycans (PG) from the surface of the cartilage layers. To investigate this early cartilage destruction which is mainly mediated by aggrecanases, total knee joint sections were stained with safranin O. Loss of red staining (a measure for PG loss) was scored in various cartilage layers of the knee joint (medial and lateral femur, tibia and patella) using an arbitrary scale from 0-3. At day 3 after IFN-γ-stimulated arthritis induction, PG loss in arthritic WT knee joints varied from 1 in the patella up to 3 in the lateral and medial femur. At day 7 after ICA induction, nearly maximal PG loss was found in all cartilage layers investigated. Comparable PG depletion was found in arthritic P47phox$^{-/-}$ knee joints both at day 3 and day 7 after arthritis induction (Figure 4A and B), suggesting that NADPH-oxidase driven oxygen radicals do not alter aggrecanase-activity, which is responsible for PG loss. Arthritic knee joints which were not injected with AdIFN-γ also showed maximal PG loss which was not different between the two strains (data not shown).

Oxygen radicals aggravate MMP-mediated cartilage destruction during IFN-γ-stimulated ICA

As PG loss was not different between P47phox$^{-/-}$ and WT controls, we additionally investigated the more severe cartilage

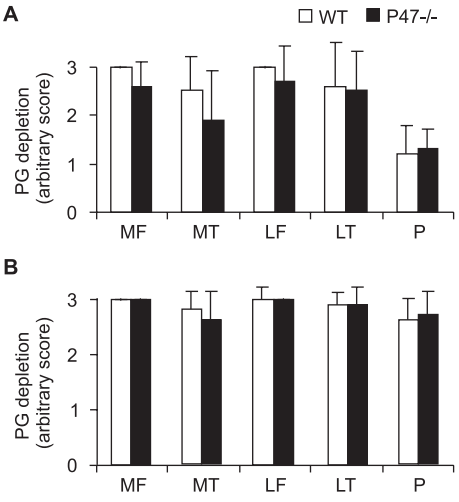

Figure 4
PG depletion in cartilage layers of total knee joint sections 3 (A) and 7 (B) days after induction of IFN-γ-stimulated ICA in P47phox$^{-/-}$ and their WT controls. PG depletion was scored as loss of red staining in tibia, femur, and patella using an arbitrary scale from 0-3. No significant difference in PG loss was found at day 3 (A) or day 7 (B) between wild-type controls and P47phox$^{-/-}$ mice. Data are expressed as loss of red staining when compared to control cartilage layers and represent the mean ± SD of 8 mice. Significance was tested using the Wilcoxon rank test (*, P<0.05). MT, medial tibia; LT,lateral tibia; LF, lateral femur; MT, medial tibia; MF,medial femur; P,patella.

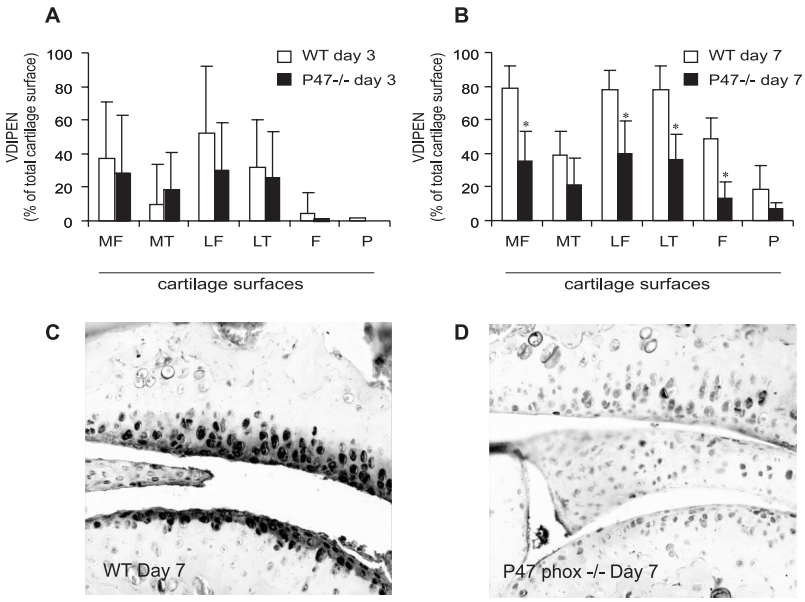

Figure 5
VDIPEN staining in knee joints of P47phox$^{-/-}$ and their wild-type controls, 3 (A) and 7 (B) days after IFN-γ-stimulated ICA. VDIPEN staining was significantly lower at day 7 in lateral and medial femur and lateral tibia of P47phox$^{-/-}$ mice (B and photographs D versus C). Data represent the mean ± SD of 8mice. Significance was tested using the Wilcoxon rank test (*, P<0.05). MT, medial tibia; LT, lateral tibia; MF, medial femur; LF, lateral femur; P, Patella. Original magnification, x 100.

matrix destruction mediated by MMPs. For this purpose, the amount of MMP-specific neoepitope VDIPEN expressed within various cartilage layers within the knee joint was determined by immunostaining using specific anti-VDIPEN antibodies. A progressive amount of VDIPEN staining was observed at day 7 when compared to day 3 in cartilage layers of IFN-γ-stimulated arthritic WT knee joints but not in P47phox$^{-/-}$ knee joints (Figure 5A and B).

In WT controls, the amount of VDIPEN staining varied from 5% in the patella up to 55% in the lateral femur, 3 days after arthritis induction. In P47phox$^{-/-}$ mice, VDIPEN staining in various cartilage layers was comparable to WT controls at that time-point (Figure 5A). At day 7 after arthritis induction, VDIPEN staining varied between 10 and 80% in WT controls. Interestingly, in knee joints of arthritic P47phox$^{-/-}$, VDIPEN staining was significantly lower in lateral femur, medial femur and lateral tibia (respectively 50%, 60% and 50% reduction) (Figure 5B and C versus D) and values were similar as found at day 3.

Oxygen radicals downregulate MMP mRNA levels within the inflamed synovium during IFN-γ-stimulated ICA

An important source of MMPs involved in cartilage destruction may be derived from the inflamed synovium. To investigate whether oxygen radicals

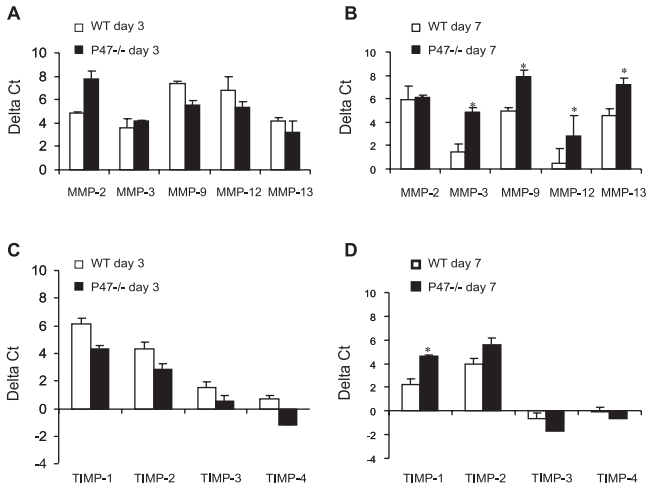

Figure 6
Expression of mRNA levels of various MMPs (MMP-2,-3,-9,-12, and -13) and their inhibitors (TIMP-1,-2,-3,and -4) in synovia of arthritic P47phox$^{-/-}$ and WT knee joints. Synovium was isolated 3 and 7 days after IFN-γ-stimulated arthritis. The cycle threshold (Ct) value of the various MMP and TIMP genes was corrected for GAPDH content. Note that at day 7, significant elevated levels of MMP-3, -9, and -13 were found in P47phox$^{-/-}$ mice when compared with controls (B). TIMP-1 and 2 were also somewhat elevated, although to a lesser extend than MMPs (D). No significant differences were found at day 3 (A and C). Significance was tested using the Wilcoxon rank test (*, P<0.05).

alter the expression of MMPs within the inflamed synovium, well-defined synovial specimen were isolated at days 3 and 7 after arthritis induction and mRNA levels of various MMPs (MMP-2,-3,-9,-12, and -13) and their inhibitors (TIMP-1,-2,-3, and -4) were determined using quantitative RT-PCR. At day 3 after IFN-γ-accelerated ICA, moderate expression (ΔCt 4 to 8 cycles) of all MMPs was measured in WT mice. TIMP-1 and -2 were moderately expressed, in contrast to TIMP-3 and -4 which were almost absent. At day 7, MMP-2, -9, and -13 were still highly expressed and only MMP-3 and 12 were significantly downregulated (Figure 6A and B). TIMP-1, but not TIMP-2, was significantly lower at day 7 (Figure 6C and D). In synovium of day 3 arthritic P47phox$^{-/-}$ knee joints, MMP-2 was higher and MMP-9 lower when compared with WT controls (Figure 6A). TIMP-1 and -2 expression was somewhat lower, not reaching significance (Figure 6C). At day 7, P47phox$^{-/-}$, when compared with WT controls, showed a significant elevation of MMP-3, -9, and -13 (Figure 6B), whereas also TIMP-1 and -2 were somewhat increased (Figure 6D).

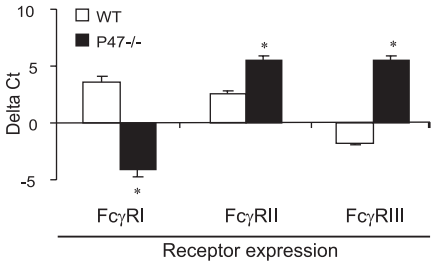

Figure 7
Expression of mRNA levels of various FcγR (FcγRI, -II, and -III) in synovia of arthritic P47phox$^{-/-}$ and WT knee joints. Synovium was isolated 7 days after IFN-γ-stimulated arthritis. The cycle threshold (Ct) value of the various FcγR genes was corrected for GAPDH content and for values in naive knee joints. Note the significant lower levels of FcγRI and elevated levels of FcγRII and III in P47phox$^{-/-}$ mice when compared with controls. Significance was tested using the Wilcoxon rank test (*, P<0.05).

Oxygen radicals increase FcγRI and decrease FcγRII and III during IFN-γ-stimulated ICA

In previous studies, we have found that activating FcγR (mainly FcγRI) are important in the activation step of latent

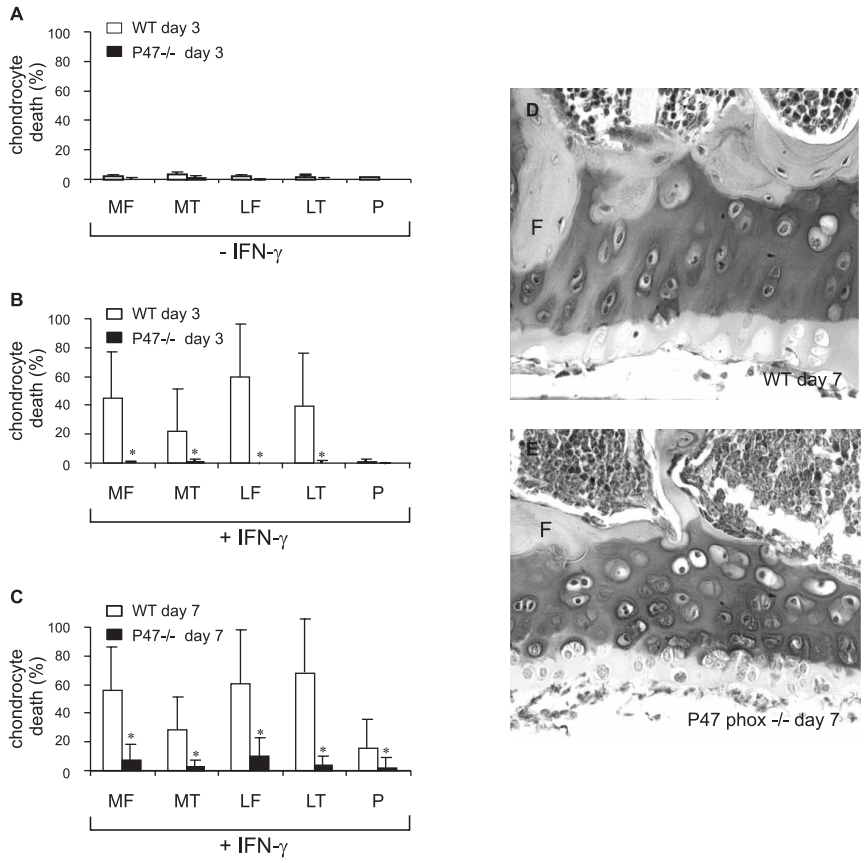

Figure 8
Chondrocyte death in various cartilage layers of knee joints of P47phox$^{-/-}$ and their WT controls at day 3 after ICA induction (A) and at day 3 (B) and day 7 (C) after IFN-γ-stimulated ICA. Chondrocyte death was expressed as percentage of empty lacunae. Note that without IFN-γ, no chondrocyte is observed. At day 7 after induction of IFN-γ-stimulated arthritis, in WT controls chondrocyte death was clearly present, whereas in P47phox$^{-/-}$ chondrocyte death was completely absent (C and E versus control D). Significance was tested using the Wilcoxon rank test (*, P<0.05). MT, medial tibia; LT,lateral tibia; LF, lateral femur; MT, medial tibia; MF, medial femur; P, patella; F, femur. Original magnification, x 400.

MMPs [13]. To further investigate whether oxygen radicals are involved in the regulation of FcγR expression, mRNA levels of the three FcγR classes were determined in synovia isolated 7 days after induction of IFN-γ-stimulated ICA. In WT mice, FcγRI and II were upregulated (ΔCt 4 and 2, respectively), whereas expression of FcγRIII was decreased (ΔCt 2) when compared to naive knee joints. In P47phox$^{-/-}$, FcγRI was downregulated (ΔCt 4), whereas FcγRII and III were both strongly upregulated (ΔCt 6) (Figure 7).

Oxygen radicals determine chondrocyte death during IFN-γ-stimulated ICA

Apart from MMP-mediated cartilage destruction, chondrocyte death is an important parameter of severe car-

tilage destruction. In previous studies we found that during IFN-γ-stimulated ICA, chondrocyte death was dependent on FcγRI. As binding of IC to FcγRI results in substantial oxygen radical production [14], which subsequently leads to a significant up-regulation of this receptor, we further investigated whether NADPH-oxidase driven oxygen radical production indeed mediates chondrocyte death within this model. The amount of empty lacunae (expressing chondrocyte death) within various cartilage layers of the knee joint was determined and expressed as a percentage of the total amount of chondrocytes present. Without IFN-γ overexpression, chondrocyte death was not found in WT and P47phox$^{-/-}$ arthritic knee joints (Figure 8A). In contrast, when AdIFN-γ was injected before arthritis induction, chondrocyte death was tremendously increased to 40 and 60% in the lateral and medial femur, respectively, and to 20 and 40% in the lateral and medial tibia, respectively, at day 3 in WT mice (Figure 8B). At day 7, chondrocyte death was even higher (between 60-70% and 20-70% in femur and tibia, respectively) (Figure 8C and D). Interestingly, arthritic knee joints of IFN-γ-stimulated P47phox$^{-/-}$, although joint inflammation was comparable to that found in WT mice, chondrocyte death was completely absent at day 3 and very low at day 7 (between 2-5% and 5-8% in tibia and femur, respectively (Figure 8B, C, and E). Absence of chondrocytes may lead to cartilage erosion. However, at day 7 after IFN-γ-stimulated ICA, erosion was still mild in cartilage layers of WT arthritic knee joints. Erosion pits were only found in the superficial layers of the medial and lateral tibia. Furthermore, clear attachment of macrophages to the cartilage surface was observed. Cartilage layers in arthritic P47phox$^{-/-}$ knee joints showed similar attachment of macrophages and mild erosion, although chondrocyte death was absent (Figure 8E).

Discussion

In the present study, we find that in the absence of NADPH-oxidase generated oxygen radicals, IC-mediated joint inflammation was significantly enhanced. This might be due to a disturbed IC-clearance, since removal of ICs from the joint determines the severity of arthritis [28]. This is in line with a previous study, which showed that oxygen radicals are crucial in clearance of foreign particles like cell walls of micro-organisms [29]. In a previous study, we have found that injection of zymosan directly into the knee joint of P47phox$^{-/-}$ mice caused a strongly elevated joint inflammation due to retarded clearance and resulted in prominent granuloma formation within the synovia of these mice [30]. In the present study, we find that IFN-γ overexpression in the knee joint of P47phox$^{-/-}$ before ICA induction prevented the raise in joint inflammation and no granuloma formation was found. IFN-γ is a potent inducer of receptors involved in phagocytosis like FcγR and complement receptors and might lead to an efficient removal of ICs, responsible for on-going arthritis within the knee joints of P47phox$^{-/-}$ mice. Macrophages form the dominant cell type within this model and these cells express large amounts of FcγR which are largely responsible for IC clearance, but also for activation of

the lining cells which drive arthritis [31]. Interestingly, synovial expression of the inhibitory FcγRII, which has been shown to be the dominant FcγR involved in IC clearance [32] was upregulated in the synovium of IFN-γ-stimulated arthritic P47phox$^{-/-}$ knee joints. Because FcγRII is not dependent on oxygen radicals for efficient clearance, increase of FcγRII expression may lead to a more efficient IC-clearance.

Although the amount of infiltrated macrophages was not different between arthritic P47phox$^{-/-}$ and WT knee joints, destruction of the cartilage matrix by metalloproteinases was lower in the absence of oxygen radicals. Cytokines like IL-1 and TNF-α activate chondrocytes and synoviocytes to produce MMPs which are released in an inactive form. These latent enzymes need an activation step before they can degrade the cartilage matrix. MMP-3 is the crucial MMP involved in activation of MMP-13, which forms the rate-limiting MMP in degradation of the collagen type II matrix resulting in erosion of the cartilage matrix [5]. IFN-γ overexpression strongly increased MMP expression in the synovium. This may be regulated directly by IFN-γ or indirectly by up-regulation of FcγR expression and their subsequent activation by ICs. In the present study we find that inflamed synovia of IFN-γ-stimulated arthritic P47phox$^{-/-}$ knee joints showed a strong up-regulation of various MMPs like MMP-3, -9, -12, and -13. TIMP-1 and TIMP-2 were only marginally up-regulated, whereas TIMP-3 and -4 were completely absent. This suggests that oxygen radicals inhibit gene expression of MMPs. As MMP-mediated cartilage destruction was lower in arthritic P47phox$^{-/-}$ knee joints, whereas MMP expression in the synovium appeared higher, this may indicate that oxygen radicals are also involved in their activation. Oxygen radicals have earlier been shown to activate latent MMP like MMP-2 [17]. In the present study we also find that oxygen radicals up-regulate FcγRI expression. Binding of ICs to FcγRI leads to increased oxygen radical production [14] and may form an amplification step in the activation of pro-MMPs.

An interesting difference in contribution of oxygen radicals to MMP-mediated cartilage damage in P47phox$^{-/-}$ mice was found between arthritis induced by zymosan (ZIA) or by ICs. During IFN-γ-stimulated ICA, oxygen radicals enhance, whereas during ZIA, they inhibit MMP-mediated cartilage damage. An explanation for this discrepancy may be the cell type involved in mediating cartilage destruction. During ZIA, a high numbers of PMNs infiltrate into the joint. These cells do not attach to the cartilage surface and release large amounts of enzymes. Crucial enzymes released by PMNs are elastase and cathepsin G, which are highly capable of penetrating the cartilage due to their highly positive charge and are then able to stimulate pro-MMP into their active form causing VDIPEN neoepitopes [33]. Under normal circumstances elastase activity is inhibited by synovial fluid inhibitors like α2-macroglobulin and no VDIPEN staining can be detected within the cartilage layers [4]. However, in the absence of oxygen radicals the number of infiltrated PMNs was strongly increased during ZIA [30] and the amount of elastase may then overrule the inhibiting capacity

of α2-macroglobulin in the synovial fluid. In contrast to ZIA, during IFN-γ-stimulated ICA the dominant infiltrating cell is the macrophage which strongly attaches to the surface of the cartilage. Production of oxygen radicals such as hydrogen peroxide generated after stimulation of FcγR by ICs [14] and the presence of superoxide dismutase, may then be of crucial importance in regulating activation of pro-MMPs in the cartilage matrix. Hydrogen peroxide has a relatively long half-live and is able to activate pro-MMPs [17]. Synovial fluid contains large amounts of inhibitors of hydrogen peroxide like catalase [34]. However, due to the close proximity of the activated macrophage to the cartilage surface, hydrogen peroxide may escape from this inhibitor, which due to its large size (240kD) is not able to penetrate into the cartilage matrix [35].

Another parameter of severe cartilage destruction is chondrocyte death, which was completely abrogated in the absence of NADPH-oxidase driven oxygen radicals. Chondrocyte death may be mediated by oxygen radicals released by the chondrocyte itself or by the inflamed synovium. Chondrocytes do express NADPH-oxidase [36] and cytokines such as IL-1 are potent inducers of oxygen radicals in chondrocytes [37]. Production of intra-cellular hydrogen peroxide inside the chondrocyte can cause disruption of the mitochondrial membrane leading to apoptosis [38]. However, previous studies in our lab have shown that FcγR-activated synovium is of crucial importance in mediating chondrocyte death [39]. During IFN-γ-stimulated ICA, the infiltrated macrophages become activated by ICs mainly via FcγR. In the mouse knee joint, FcγRI is exclusively expressed by macrophages, and not by neutrophils, and becomes strongly upregulated by IFN-γ. Binding of IC to particularly FcγRI, and in lesser extend to FcγRIII, leads to production of oxygen radicals. Apart from FcγRI stimulation, IFN-γ itself has been shown to upregulate P91 and P47 components of the NADPH-oxidase complex, which may contribute to the enhanced superoxide generation [40]. Besides the NADPH-oxidase pathway, P47phox$^{-/-}$ may produce oxygen radicals by alternate pathways [23]. However, IFN-γ alone had no effect on chondrocyte death. Moreover, it has been shown that IFN-γ does not upregulate alternative ways of oxygen radical production in P47phox$^{-/-}$ mice [23]. This indicates that chondrocyte death is completely mediated via the NADPH-oxidase complex. IFN-γ induces up-regulation of NADPH-oxidase components and FcγRI [41], and stimulation of FcγRI by ICs may additionally lead to an enormous increase in oxygen radical production mediating cartilage destruction. Hydrogen peroxide may again be the most plausible oxygen species mediating chondrocyte death. Hydrogen peroxide can easily penetrate through cell membranes. Earlier studies have shown that hydrogen peroxide, when injected into mouse knee joints, was able to induce considerable chondrocyte death, which may be induced by apoptosis [42]. Hydrogen peroxide activates the opening of the mitochondrial permeability transition pore and the release of cytochrome C [43]. In the cytoplasm, cytochrome C in combination with Apaf-1 activates caspase-9 leading to the activation of caspase-3 and subsequent apoptosis [44].

NADPH-oxidase and p47phox phos-

phorylation is strongly increased in leucocytes derived from synovial fluid of RA patients [45]. Cytokines such as IFN-γ are potent candidates for up-regulation of NADPH-oxidase [41]. Moreover, ICs are found in considerable amounts in joints of many RA patients. These ICs may be responsible for a large part of NADPH-oxidase activation via FcγRI stimulation, resulting in major amounts of oxygen radicals. The latter may mediate part of the severe cartilage destruction. As FcγRI-mediated oxygen radical production may play a major role in mediating cartilage destruction during arthritis, this receptor may form a crucial target in combatting this crippling disease.

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Chapter 9

Summary & Final considerations
Nederlandse samenvatting

Curriculum Vitae
List of Publications
Dankwoord
Appendix

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease, which is characterized by uncontrolled proliferation of synoviocytes and massive infiltration of leucocytes. The perpetuation of synovitis often results in erosion of cartilage and bone. Although RA is considered as an autoimmune disease, the auto-antigen is still unknown. It is thought however, that immune complexes (IC) play a dominant role in the pathogenesis of RA by activation of macrophages via binding to Fcγ receptors (FcγRs).

The aim of this thesis was to investigate the role of the individual FcγRs in joint inflammation and cartilage destruction and to study whether regulation of FcγR expression is able to modulate the severity of cartilage damage.

In **chapter 2**, we showed that in T cell-mediated antigen-induced arthritis (AIA) FcγRI and FcγRIII are redundant in mediating synovial inflammation, whereas FcγRI and not FcγRIII is the dominant activating receptor involved in severe cartilage destruction. Furthermore, we demonstrated that the inhibiting FcγRII is crucial in negative regulation of both acute and chronic inflammation and late severe cartilage damage. Differences in severity of arthritis in FcγRI$^{-/-}$ and FcγRIII$^{-/-}$ mice were not due to impaired T and B cell-mediated immunity, whereas in FcγRII$^{-/-}$ mice the humoral response was enhanced compared with their wild-type (WT) controls. Subsequently, we investigated the mechanism by which FcγRII inhibited the arthritic response (**chapter 3**). We found that in absence of activating FcγRs, FcγRII reduced arthritis not only by inhibiting activating FcγRs, but also by removal of ICs via endocytosis. AIA was induced in mice lacking all three FcγRs (FcγRI/II/III$^{-/-}$) and in mice lacking both activating FcγRI and III (FcγRI/III$^{-/-}$) in which FcγRII is still present. In FcγRI/III$^{-/-}$ mice, joint inflammation was almost absent. Remarkably, in FcγRI/II/III$^{-/-}$ mice, arthritis was tremendously increased compared with WT controls. Significant accumulation of IgG was observed only in arthritic knee joints of FcγRI/II/III$^{-/-}$ mice, suggesting that endocytosis and clearance of ICs was impaired in absence of FcγRII. In vitro studies using macrophages expressing only FcγRII (FcγRI/III$^{-/-}$) showed prominent endocytosis of preformed soluble ICs. In total absence of FcγRs (FcγRI/II/III$^{-/-}$), macrophages completely failed to endocytose ICs. Although joint inflammation was much higher in arthritic knee joints of FcγRI/II/III$^{-/-}$ mice and the infiltrated inflammatory cells were activated, severe cartilage destruction was completely prevented in contrast to the marked cartilage destruction (MMP-mediated VDIPEN expression and chondrocyte death) found in WT controls.

The individual role of FcγRs was further studied in arthritis solely provoked by ICs using the passively induced immune complex-mediated arthritis (ICA) (**chapter 4**). In FcγRIII$^{-/-}$ mice, influx and activation of inflammatory cells was largely blocked and resulted in reduced cartilage destruction expressed as MMP-mediated VDIPEN expression and chondrocyte death. In FcγRI$^{-/-}$ mice, joint inflammation was similar when compared with WT arthritic knee joints. However, chondrocyte death and VDIPEN expression were significantly lower, indicating that activation of FcγRI is crucial in cartilage damage. In FcγRII$^{-/-}$ mice, the arthritic

response was markedly elevated as both joint inflammation and irreversible cartilage damage were enhanced.

In AIA as well as ICA, FcγRI appeared to be the crucial receptor in development of severe cartilage destruction, since absence of FcγRI did not alter the inflammatory response, whereas irreversible cartilage destruction was significantly reduced. Moreover, in the T cell-mediated AIA, which is a Th1 driven disease, cartilage destruction was more severe and completely dependent on FcγRI. Th1 cells release interferon (IFN)-γ, which is known to induce marked up-regulation of FcγRI and this might explain why FcγRI becomes prominent in cartilage destruction during AIA. In **chapter 5**, we demonstrated that local overexpression of IFN-γ in the knee joint during ICA, using an adenovirus, indeed deteriorated irreversible cartilage damage. This was only found in arthritis mediated by ICs, and not when arthritis was induced by zymosan. IFN-γ did not affect the amount of inflammatory cells, but altered the composition of the cell mass as the percentage of macrophages expressing markers for a proinflammatory phenotype was elevated. FcγRI mRNA level in synovium was significantly augmented by IFN-γ. Whether this contributed to the severity of cartilage damage was studied using FcγRI$^{-/-}$ mice. Interestingly, despite IFN-γ overexpression, chondrocyte death remained low in arthritic FcγRI$^{-/-}$ knee joints, indicating that FcγRI specifically mediated chondrocyte death. On the contrary, IFN-γ enhanced MMP-mediated VDIPEN expression similarly as found in WT controls, suggesting that the activating FcγRIII is able to compensates absence of FcγRI. To elucidate the role of FcγRIII further, IFN-γ was also overexpressed in FcγRIII$^{-/-}$ mice before ICA induction (**chapter 6**). FcγRIII dependency of joint inflammation within this model was completely abrogated by IFN-γ, resulting in comparable joint inflammation in FcγRIII$^{-/-}$ mice and WT controls. Levels of macrophage-attractant MIP-1α were increased by IFN-γ and correlated with the higher percentage of macrophages found both in WT controls and FcγRIII$^{-/-}$ mice. Enhanced VDIPEN expression and chondrocyte death were also observed in FcγRIII$^{-/-}$ mice. Combining the results from chapter 5 and 6, we demonstrated that IFN-γ increased the severity of cartilage destruction mainly via up-regulation of activating FcγRs. Both activating FcγRs mediated MMP-dependent VDIPEN expression, whereas FcγRI specifically induced chondrocyte death.

In **chapter 7**, we showed that overexpression of the Th2 cytokine IL-13 reduced cartilage destruction. In arthritic knee joints injected with adenovirus expressing IL-13, the amount of inflammatory cells was elevated and higher numbers of polymorphonuclear neutrophils (PMNs) were observed. Chondrocyte death was however diminished, which correlated with downregulation of FcγRI mRNA level in synovium. MMP-mediated VDIPEN expression was also decreased, although MMP mRNA and IL-1 protein levels were enhanced. The latter suggests that IL-13 may interfere at the level of activation of pro-MMPs, instead of decreasing the amount of MMPs.

In the studies mentioned above, we demonstrated that FcγRs are of crucial

importance in the development of irreversible cartilage damage and that regulation of FcγR expression by cytokines is able to modulate the severity of cartilage destruction. Moreover, a prominent role for FcγRI was found mediating irreversible cartilage destruction. It is known that binding of ICs to FcγRI results in prominent oxygen radical production by the NADPH-oxidase complex. Whether NADPH-oxidase driven oxygen radical production is involved in development of chondrocyte death and MMP-mediated proteoglycan damage was investigated in **chapter 8**. Therefore IFN-γ-stimulated IC-arthritis was elicited in mice lacking the P47-subunit (P47phox$^{-/-}$), which is necessary for a functional NADPH-oxidase complex. Joint inflammation was similar in P47phox$^{-/-}$ mice and WT controls when IFN-γ was overexpressed. However, chondrocyte death and MMP-mediated cartilage destruction were significantly lower which was accompanied by decreased FcγRI mRNA levels in synovium. This study suggests that production of oxygen radicals via NADPH-oxidase after stimulation of activating FcγRI might contribute to chondrocyte death and MMP-mediated VDIPEN expression.

Final considerations

This thesis demonstrates that activation of FcγRs by IgG-containing ICs in the joint determines inflammation and irreversible cartilage destruction.

In many RA patients, IgG-ICs are abundantly expressed in synovial fluid, synovium and cartilage [1,2], and therefore FcγRs may be crucial in the pathology found in RA. Recently, it was found that antibodies directed against citrullinated proteins are highly specific for RA. Besides being used as diagnostic factor, these antibodies may form ICs locally in the joint, thereby activating macrophages by binding FcγRs.

In this thesis, two IC-dependent arthritis models were used to study the role of FcγRs. In selective FcγR-deficient mice, antigen-induced arthritis (AIA), regulated by both ICs and T cells, or immune complex-mediated arthritis (ICA), solely provoked by ICs, was elicited. In AIA, irreversible cartilage destruction was specifically FcγRI dependent, whereas in ICA, both activating FcγRI and III mediated cartilage destruction. The discrepancy in FcγR dependency may be explained by contribution of the T cells and their cytokines, as this is the most striking difference between these models. However, the role of antibody isotypes was not investigated. The three classes of FcγRs have different affinities for the various IgG subclasses [3,4] and the isotype of antibodies found within the arthritis model might also contribute to FcγR dependency of the model. In our passive ICA model, antibodies derived from rabbits are used, which can activate all three murine FcγRs. On the contrary, in AIA, mice are immunized resulting in antibodies of various IgG subclasses, which bind to FcγRs with different affinity. To study the role of antibodies in arthritis further, we are now developing antibodies of various isotypes directed against lysozyme, which can than be used to elucidate the individual role of of the individual isotypes in eliciting arthritis and cartilage destruction. How-

ever, expression of FcγRs present in the joint remains of crucial importance in the arthritic process.

The inhibiting FcγRII negatively regulated joint inflammation and cartilage damage both in AIA and ICA, which is in line with studies describing the inhibiting role of FcγRII in collagen-induced arthritis [5,6]. FcγRII not only reduced the arthritic response by inhibiting the activating FcγRs, a function generally highlighted. We now demonstrated for the first time in vivo that in absence of activating FcγRs, the inhibiting FcγRII still functions as an important downregulator of synovial inflammation by clearance of immune complexes. These results indicate that up-regulation of this inhibiting Fc receptor may repress the arthritic response and development of cartilage destruction, and therefore may be a very promising approach for therapy. Increased expression of FcγRII can be achieved by cytokines such as IL-4 and IL-13 [7], or by local gene transfer of FcγRII using adenoviral constructs [8]. Recently, we developed an adenovirus expressing the inhibiting FcγRII and tested this construct in vitro. We found that FcγRIIb expressed by the adenovirus was functional, since non-phagocytic COS-1 cells were able to phagocytose IgG-coated sheep erythrocytes when transfected. The effects of FcγRII overexpression during the arthritic response is momentarily under investigation.

In mouse, detection of FcγR protein expression is still difficult due to lack of specific antibodies recognizing individual FcγRs. Therefore, regulation of FcγR expression by cytokines in the knee joint was studied on mRNA level. FcγRII and III protein can be detected by the monoclonal 2.4G2 [9], which can be used for immunohistochemistry and flowcytometer analysis. Only recently, an antibody was developed against murine FcγRI [10], which enables detection of FcγRI by flowcytometer analysis. To further extend our knowledge of regulation of FcγR expression locally in the knee joint during arthritis, specific antibodies for immunohistochemistry need to be developed.

As FcγRI is crucial in development of irreversible cartilage destruction in experimental T cell dependent IC-mediated arthritis, it will be interesting to investigate the contribution of this receptor in cartilage destruction found in RA patients. Recently, we demonstrated in our lab, that expression of both FcγRII and III in RA synovium correlated with inflammation [11]. This link between FcγRIII expression in RA synovium and inflammation was also found in experimental arthritis, as FcγRIII is the dominant receptor mediating joint inflammation in the passive IC-arthritis. Cartilage destruction in RA affected joints can be measured by detection of cartilage degradation products like type II collagen in synovial fluids [12] or by cartilage oligomeric matrix protein (COMP) levels in serum [13]. To study whether FcγRI is also involved in cartilage destruction during RA, FcγRI expression in RA synovium or on inflammatory cells present in synovial fluid can be correlated to cartilage degradation products present in synovial fluid or serum.

In this thesis, it was found that activating FcγRs are crucial in the development of cartilage destruction. Moreover, a specific role for FcγRI was found mediating chondrocyte death. It remains to be elu-

cidated by what mechanisms activation of FcγRs induces cartilage destruction, and whether there are differences in FcγRI and FcγRIII signaling. Signaling of Fc Rs involved in phagocytosis is extensively studied, however, signal transduction resulting in MMP production or other factors involved in cartilage damage remains to be elucidated.To investigate whether there are differences in signaling between the two activating FcγRs, macrophages derived from FcγRI or FcγRIII deficient mice can be used. Differences in gene expression after stimulation with ICs can be detected on mRNA level using gene-analysis, a technique by which forty-five thousand genes are screened [14]. Recently, kinase arrays are developed which facilitates the analysis of phosphorylation of proteins, hereby giving information on signal pathways triggered after FcγR activation [15]. These techniques can be used to gain more insight in mechanisms specifically involved in cartilage degradation, triggered after activation of FcγRI. Further characterization of cartilage destruction processes activated after stimulation via FcγRs is of high interest as new target proteins can be discovered that allow us to specifically block cartilage destruction, without disturbance of the phagocytotic capacity of FcγRs which is necessary for adequate clearance of ICs.

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Reumatoïde artritis (RA) is een chronische gewrichtsontsteking, die vaak gepaard gaat met ernstige destructie van kraakbeen en bot, wat uiteindelijk kan leiden tot blijvende invaliditeit. RA komt voor bij ongeveer 1% van de wereldbevolking en het ziekteverloop wordt gekenmerkt door actieve en rustige perioden. Hoewel de oorzaak van RA nog steeds niet bekend is, wordt het beschouwd als een auto-immuunziekte waarbij een afweerreactie tegen lichaamseigen stoffen (auto-antigenen) optreedt. Nadere analyse van ontstoken gewrichten wijst uit dat er verschillende celtypen aanwezig zijn, zoals macrofagen, T cellen en plasmacellen. Ook worden er in de gewrichten antilichaam-antigeen complexen (immuuncomplexen, ICs) gevonden. Er zijn verschillende typen antilichamen bekend, te weten IgA, IgM, IgE, IgD en IgG en hierdoor kunnen er verschillende soorten ICs worden gevormd. In het gewricht van RA patiënten worden veel IgG-bevattende ICs gevonden. Deze IgG-ICs activeren bepaalde cellen, de macrofagen, hetgeen leidt tot productie van ontstekingsmediatoren zoals interleukine 1 (IL-1), tumor necrosis factor (TNF)-α en enzymen zoals matrix metalloproteinasen (MMPs). IgG-ICs kunnen aan macrofagen binden via speciale eiwitten aanwezig op het celoppervlak, de Fcγ receptoren (FcγRs). Er zijn drie verschillende typen FcγRs, genaamd FcγRI, II, en III. Binding van ICs aan FcγRI of FcγRIII leidt tot activatie van macrofagen, terwijl binding aan FcγRII de macrofaag juist inactiveert.

Om het RA proces te bestuderen wordt er gebruik gemaakt van proefdiermodellen. Een van die modellen is immuuncomplex artritis (ICA). In dit model worden muizen ingespoten met antilichamen die het eiwit lysozym herkennen. Vervolgens wordt lysozym in het kniegewricht geïnjecteerd, waarin dan locaal ICs ontstaan. Deze ICs activeren de macrofagen die de binnenbekleding van het gewricht vormen, hetgeen leidt tot migratie van ontstekingscellen naar het gewricht. Een tweede experimenteel model is de antigeen-geïnduceerde artritis (AIA), waarbij niet alleen ICs een rol spelen maar ook T cellen. Muizen worden geïmmuniseerd tegen een eiwit (mBSA) waardoor er antilichamen en T cellen die dit eiwit herkennen worden geproduceerd. Na drie weken wordt het eiwit in het kniegewricht geïnjecteerd, dat vervolgens door binding aan antilichamen ICs vormt en een ontstekingsreactie in gang zet.

In beide modellen zal de ontsteking in het gewricht leiden tot onherstelbare afbraak van kraakbeen. Deze ernstige kraakbeenschade wordt niet gevonden wanneer artritis wordt geïnduceerd door injectie van gist- of bacterie-deeltjes. Dit wijst erop dat ICs noodzakelijk lijken te zijn in het ontstaan van onherstelbare kraakbeenschade. Kraakbeen bestaat uit een matrix van proteoglycanen en collagenen die geproduceerd worden door kraakbeencellen, de chondrocyten. Kraakbeenschade begint met verlies van proteoglycanen uit de kraakbeen matrix. Wanneer het proces van kraakbeenschade voortduurt worden collagenen afgebroken en gaan chondrocyten dood. Dit kan uiteindelijk leiden tot het verdwijnen van de kraakbeenlaag (erosie).

In deze studie werd de functie van de drie verschillende FcγRs in het ontstaan van gewrichtsontsteking en kraakbeenschade tijdens artritis bestudeerd. Ook

werd er onderzocht of veranderingen in FcγR expressie de ernst van kraakbeenschade kan beïnvloeden en via welk mechanisme binding van ICs aan FcγRs kan leiden tot onherstelbare kraakbeenschade.

Allereerst werd de rol van de drie individuele FcγRs in een T cel afhankelijk immuuncomplex artritis model (antigeen-geïnduceerde artritis, (AIA)) bestudeerd (**hoofdstuk 2)**. Hiervoor werd gebruik gemaakt van muizen die specifiek FcγRI, FcγRII, of FcγRIII missen (FcγRI$^{-/-}$, FcγRII$^{-/-}$, FcγRIII$^{-/-}$ muizen), doordat in het genoom deze genen zijn uitgeschakeld. Ondanks afwezigheid van FcγRI of FcγRIII (FcγRI$^{-/-}$ en FcγRIII$^{-/-}$), resulteerde injectie van mBSA in een ontstekingsreactie vergelijkbaar met die in controle muizen. Onherstelbare kraakbeenschade gedefinieerd als chondrocyt dood en matrix metalloproteinase (MMP) geïnduceerde neo-epitope (VDIPEN) expressie, was in FcγRI$^{-/-}$ muizen sterk verminderd. In FcγRIII$^{-/-}$ muizen was de kraakbeenschade gelijk aan die gevonden in controle muizen. Muizen zonder de remmende FcγRII ontwikkelden zowel ernstigere ontsteking als onherstelbare kraakbeenschade. Deze resultaten laten zien dat de ontstekingsreactie tijdens de AIA bepaald wordt door FcγRI en III. Onherstelbare kraakbeenschade daarentegen, is volledig afhankelijk van FcγRI. FcγRII is een belangrijke remmer van zowel ontsteking als kraakbeenschade die ontstaat tijdens AIA.

In **hoofdstuk 3** werd bestudeerd op welke manier FcγRII ontsteking en kraakbeenschade tijdens AIA remt. Hiervoor werden muizen gebruikt zonder FcγRs (FcγRI/II/III$^{-/-}$) en muizen met alleen de remmende FcγRII (FcγRI/III$^{-/-}$). In FcγRI/III$^{-/-}$ muizen met artritis waren nauwelijks ontstekingscellen te zien in het kniegewricht. Opmerkelijk was dat in FcγRI/II/III$^{-/-}$ muizen de ontsteking juist veel ernstiger was. Verder was er ophoping van IgG antilichamen in de gewrichten zichtbaar, hetgeen erop kan duiden dat ICs minder goed worden opgeruimd. Dit werd onderzocht door opname van fluorescerende ICs door macrofagen te bestuderen. Macrofagen met alleen FcγRII op het celoppervlak (FcγRI/III$^{-/-}$) konden deze ICs effectief op te nemen, terwijl macrofagen zonder FcγRs (FcγRI/II/III$^{-/-}$) geen ICs opnamen. Ondanks de ernstige ontsteking in kniegewrichten van FcγRI/II/III$^{-/-}$ muizen, was ernstige destructie van het kraakbeen volledig afwezig. Dit laatste bewijst opnieuw dat activerende FcγRs cruciaal zijn in het ontstaan van kraakbeenschade.

De functie van de FcγRs werd daarna onderzocht in een passief geïnduceerd immuuncomplex artritis model (ICA) (**hoofdstuk 4**). In afwezigheid van FcγRIII waren ontsteking en macrofaag activatie sterk verminderd. FcγRI speelde geen rol in ontsteking. Ook in dit model resulteerde afwezigheid van de remmende receptor (FcγRII$^{-/-}$) in een ernstigere ontsteking. Onherstelbare kraakbeenschade was zowel in FcγRI$^{-/-}$ als in FcγRIII$^{-/-}$ muizen sterk verminderd, terwijl deze schade in FcγRII$^{-/-}$ muizen juist enorm verergerde.

In hoofdstuk 2 en 4 laten we zien dat afwezigheid van FcγRI zowel in AIA als in ICA, resulteert in sterk verminderde onherstelbare kraakbeenschade. In het

T cel afhankelijke artritis model was de irreversibele kraakbeenschade ernstiger en compleet afhankelijk van FcγRI. Een verklaring voor het laatste kan zijn dat T cellen cytokinen produceren die de expressie van FcγRI verhogen, waardoor de bijdrage van deze receptor in het ontstaan van kraakbeenschade belangrijker wordt. Een bekend T cel cytokine dat FcγRI expressie verhoogt, is interferon (IFN)-γ. In **hoofdstuk 5** hebben we het effect van IFN-γ op ernstige kraakbeenschade bestudeerd en hebben we onderzocht welke rol FcγRI hierin speelde. IFN-γ werd tot overexpressie gebracht door injectie van een adenoviraal construct in het kniegewricht waarna ICA werd opgewekt. Door aanwezigheid van IFN-γ tijdens ICA werd de irreversibele kraakbeenschade inderdaad veel ernstiger. Dit effect van IFN-γ werd alleen gevonden in experimentele artritis geïnduceerd door ICs. Of de toename in onherstelbare kraakbeenschade werd veroorzaakt door verhoging van FcγRI expressie, werd bestudeerd in FcγRI$^{-/-}$ muizen. Ondanks aanwezigheid van IFN-γ tijdens ICA, werd er nauwelijks chondrocyt dood gevonden in FcγRI$^{-/-}$ kniegewrichten. MMP-geïnduceerde VDIPEN expressie in kniegewrichten van FcγRI$^{-/-}$ muizen werd wel verhoogd door IFN-γ. Dit laatste kan worden veroorzaakt door de activerende FcγRIII, hetgeen vervolgens werd bestudeerd in FcγRIII$^{-/-}$ muizen (**hoofdstuk 6**). Door aanwezigheid van IFN-γ tijdens ICA, werd de ontstekingsmassa in FcγRIII$^{-/-}$ kniegewrichten vergelijkbaar aan die aanwezig in controle kniegewrichten. IFN-γ verhoogde zowel chondrocyt dood als MMP-geïnduceerde VDIPEN expressie in muizen zonder FcγRIII.

De resultaten van hoofdstuk 5 en 6 laten zien dat door aanwezigheid van het Th1 cytokine IFN-γ, irreversibele kraakbeenschade ernstiger wordt tijdens IC afhankelijke artritis. Verder werd gevonden dat FcγRI en III betrokken zijn bij MMP-geïnduceerde VDIPEN expressie. Chondrocyt dood daarentegen is geheel afhankelijk van FcγRI.

Naast Th1 cytokinen zijn er ook Th2 cytokinen. Deze cytokinen hebben beschermende eigenschappen. In **hoofdstuk 7** werd onderzocht welk effect het Th2 cytokine IL-13 op ernstige kraakbeenschade heeft wanneer het tijdens artritis tot overexpressie werd gebracht. Een adenoviraal construct coderend voor IL-13, werd ingespoten in het kniegewricht, een dag voor ICA inductie. Ondanks dat beschreven is dat IL-13 ontsteking remt, verhoogde IL-13 de hoeveelheid ontstekingscellen in het gewricht. Echter, chondrocyt dood werd verminderd door IL-13 en ook werd er een verlaagde expressie van FcγRI in synovium gevonden. Overexpressie van IL-13 reduceerde eveneens MMP-geïnduceerde VDIPEN expressie in de kraakbeenmatrix. Dit kan veroorzaakt zijn door een verlaging van de MMP productie. Het tegenovergestelde werd gevonden, aangezien de hoeveelheid MMPs in het synovium juist verhoogd was. MMPs kunnen alleen kraakbeenschade veroorzaken nadat ze zijn geactiveerd. Een mogelijkheid is dat IL-13 de activering van MMPs remt, hetgeen resulteert in verlaging van MMP geïnduceerde kraakbeenschade.

De hierboven beschreven studies laten zien dat FcγRs belangrijk zijn in het ontstaan van ontsteking en kraakbeenschade tijdens artritis. De ernst van kraakbeenschade kan worden beïnvloed

via regulatie van FcγR expressie door cytokinen. Een belangrijke rol voor FcγRI werd gevonden in het ontstaan van chondrocytdood. Een mogelijke wijze waarop binding van ICs aan FcγRI leidt tot chondrocytdood is door middel van productie van zuurstofradicalen door het NADPH-oxidase complex. In **hoofdstuk 8** werd gekeken welke rol zuurstofradicalen spelen in kraakbeendestructie tijdens artritis. Hiervoor werden muizen gebruikt die het eiwit P47 missen (P47phox$^{-/-}$ muizen) waardoor geen functioneel NADPH-oxidase complex kan worden gevormd en dus ook geen zuurstofradicalen. In kniegewrichten van deze muizen werd IFN-γ tot overexpressie gebracht tijdens ICA. Ondanks de vergelijkbare ontsteking in P47phox$^{-/-}$ en controle muizen, waren chondrocyt dood en MMP geïnduceerde VDIPEN expressie significant verlaagd in afwezigheid van zuurstofradicalen. Deze data suggereren dat productie van zuurstofradicalen via het NADPH-oxidase complex verantwoordelijk is voor een deel van de chondrocytdood en MMP geïnduceerde kraakbeenafbraak.

In dit proefschrift tonen we aan dat FcγRs een belangrijke rol spelen in het ontstaan van ernstige kraakbeenschade tijdens IC artritis. Uitschakelen van de activerende FcγRs of het verhogen van de remmende FcγR in gewrichten van RA patiënten zou kraakbeenschade kunnen verminderen. Dit zou leiden tot minder vergroeiingen, waardoor invaliditeit van RA patiënten kan worden voorkomen.

Curriculum Vitae

Karin Nabbe werd geboren op 3 september 1977 te Boxmeer. In 1995 behaalde zij haar VWO diploma op het Elzendaal College te Boxmeer. In datzelfde jaar begon zij aan de studie Medische Biologie aan de Universiteit van Utrecht. Haar bijvak stage werd doorlopen op de afdeling Pediatrische Immunologie van het Wilhelmina Kinderziekenhuis te Utrecht, onder begeleiding van Dr. Rik Brooijmans en Dr. Ger Rijkers. Hier heeft zij onderzoek gedaan naar afwijkingen in de T cel receptor bij patiënten met een primaire immuundeficiëntie. Haar hoofdvak stage doorliep zij op de Department of Bacteriological Research & Development van Intervet International BV te Boxmeer, onder de begeleiding van Dr. Ir. Piet Nuijten en Prof. Dr. Wim Gaastra van de Faculteit Diergeneeskunde van de Universiteit Utrecht. In het onderzoek dat zij verrichtte werd het effect van DNA vaccinatie op de immuunrespons bij kippen bestudeerd. In februari 2000 werd het doctoraal examen met goed gevolg afgelegd.

Van februari 2000 tot februari 2005 was zij werkzaam als assistent in opleiding op het onderzoekslaboratorium Experimentele Reumatologie verbonden aan het UMC St. Radboud. Gedurende deze periode werd het onderzoek verricht wat ten grondslag ligt aan de resultaten beschreven in dit proefschrift. In 2001 won zij de Young Investigators Award op de IAIS (International Association of Inflammation Societies) te Edinburgh, Schotland. Tevens kreeg zij in 2003 een "Best Abstract Award" op de EULAR (European League against Rheumatism) in Lissabon, Portugal en won ze een Young Investigators Travel Award voor de IAIS in Vancouver, Canada.

Momenteel is zij nog steeds werkzaam als post-doc onderzoeker op dezelfde afdeling, alwaar zij onderzoek doet naar de intracellulaire processen welke in gang worden gezet na FcγR activatie.

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8. **Van Lent PLEM, Nabbe KC, Blom AB, Sloetjes AW, Holthuysen AEM, Kolls JK, van de Loo FAJ, Holland SM, van den Berg WB**: NADPH-oxidase driven oxygen radical production determines chondrocyte death and partly regulates metalloproteinase-mediated cartilage matrix degradation during interferon-γ-stimulated immune complex-mediated arthritis. *Arthritis Res Ther*, conditionally accepted
9. **Radstake TRDJ, Nabbe KC, Roelofs MF, Oosterlaar A, van Lieshout AWT, Barrera P, van Lent PLEM, van den Berg WB**: Dendritic cells from RA patients lack the IL-13 mediated increase of FcγRII expression which has clear functional consequences. *Ann Rheum Dis*, conditionally accepted

Dit is het dan! Het laatste schrijfwerk voor mijn proefschrift, en dit is waarschijnlijk het meest gelezen stuk. Mijn onderzoek is dankzij steun, vertrouwen en enthousiasme van veel mensen tot stand gekomen. Graag wil ik alvast bij deze iedereen hiervoor hartelijk danken!

Allereerst Wim, bij mijn artikelen stond je altijd als laatste, maar nu als eerste in mijn dankwoord. Ondanks je drukke werkschema had je altijd tijd voor het kritisch bekijken van artikelen, beantwoorden van vragen of andere zaken. Op allerlei luchthavens werden mailtjes gelezen en beantwoord. Verder stimuleerde je het bezoeken van congressen, zodoende heb ik in deze afgelopen jaren heel veel van de wereld mogen zien. Bedankt voor alles!

Maar natuurlijk is mijn dank ook groot aan mijn begeleider Peter. We hebben het dan toch mooi geflikt, het is af! Bedankt voor je grenzeloze enthousiasme waardoor ik ook in de moeilijkere tijden dingen positief kon blijven zien en ook voor het "eindeloos" nakijken van mijn stukken. We gaan verder met de Fcγ receptoren alleen duiken we nu de cel in. Hopelijk vinden we ook nu weer leuke resultaten.

Mijn mede-AIO-er en nu post-doc-er Arjen (Arjeni). Vanaf dag 1 ging de samenwerking al goed. Samen op het kamertje op de eerste verdieping (natuurlijk met roomie Alwin), waar ik al mijn gevoel voor orde kwijtraakte en ik ben bang dat ik "Redemption Song" me de rest van mijn leven zal blijven herinneren. Bedankt voor het fungeren als vraagbaak over de Fcγ receptor, andere vragen over het AIO zijn, maar vooral voor de gezellige tijd, niet alleen op het lab, maar ook daarbuiten.

Ook bedank ik natuurlijk Annet (Sloettie). We zijn tegelijkertijd begonnen op de afdeling en het is altijd fijn om samen nieuw te zijn. Af en toe moest er even "geklaagd" worden over het een en ander, zodat we er daarna hard om konden lachen en weer met frisse moed aan de slag konden. Op het nieuwe lab kwamen we gelukkig wat dichter bij elkaar te zitten. Helaas is het viruswerk niet meer in het boekje gekomen, ondanks de leuke resultaten in vitro. Ongetwijfeld gaan we samen nog verder onderzoek hieraan doen, zodat we het nog mooi kunnen afronden.

Last but not least uit groepje 4, Astrid (Strudel). Mijn steun en toeverlaat met het dierenwerk. We hebben gezellig wat afgekletst op het dierenlab en heel wat experimenten gedaan. Anderhalf jaar geleden ben je weggegaan op ons lab, maar je bent nog steeds helemaal op de hoogte van alles (dankzij gezellig even eten bij Riva, of ergens anders waar jij studentenkorting krijgt). Ik vind het fijn dat je me nog een keer zult steunen als mijn paranimf.

Gratis hulp is altijd welkom en mijn studenten hebben me goed geholpen. Leonie en Carlijn hebben heel wat afgestoeid met zuurstofradicalen en FcγR expressie. Dit werk zal zeker nog gaan leiden tot een mooi artikel. Dames bedankt!!! Jullie hebben nu een opvolger, namelijk Ben, die verder gaat met het werk.

Ook wil ik graag Sjef bedanken, mijn copromotor uit Leiden. Zonder jouw muizen was dit proefschrift er niet geweest. Bedankt voor de leerzame werkbesprekingen in de stationsrestauratie van Utrecht. Deze besprekingen vonden plaats onder het genot van een cappucino en een lekkere muffin, samen met Peter Boross. Ja Peter, ik ga je gewoon in het nederlands bedanken. Ik wens je heel veel succes met de afronding van jouw onderzoek. Hopelijk zal je CIA experiment nu succesvol verlopen met mooie resultaten.

Dan Ruben. Samen begonnen op het lab, samen effe een peukje doen, samen op congres (EULAR, IAIS, ACR), even naar de aesculaaf... Bedankt voor alle gezelligheid, maar wat ik zeker niet zal vergeten zijn de laatste weken (maanden). Toen was je meer "help-desk"- Ruben. Vragen over corel draw, het lay-out programma, etc. Ruben wist raad en stond paraat. Bedankt Ruub!!

Het is al een tijdje geleden, maar toen ik aan mijn onderzoek begon waren de A's aan de "macht". Alwin, mijn ex-roomie van het NIG gebouw en Andrew (H.P.). Als het vrijdag was, nou dan had je een probleem. IJs in je nek, je gezicht ingekleurd met markeerstift, of de waterkraan op je gericht. Natuurlijk was daar ook nog Alfons. Dankzij Alfons is mijn nijlpaardenverzameling op het lab enorm uitgebreid. Zag Alfons een nijlpaardje, dan moest hij het meenemen voor me. A'tjes bedankt voor de gezellige tijd op het lab!!

In die tijd was Marleen mijn mede-AIO-ster. Bedankt voor de gezelligheid op het lab, maar ook naast het werk hebben we leuke dingen gedaan. Survival in de Ardennen en de overnachting in dat tuinhuisje met jouw bruiloft zal ik nooit meer vergeten.

Nu is er een tijd aangebroken dat de dames overheersen. Marije, Esmeralda, Mieke en Shahla vormen nu de kern van junior-onderzoekers. Ik zal de retraites in Papendal met jullie wel missen hoor!!! Ik wens jullie heel veel succes met jullie onderzoek.

Dan de werkgroepleiders Peter (vd K), Leo en Fons. Bedankt voor jullie feedback tijdens de werkbesprekingen. Met Leo ging de band nog iets "verder", aangezien we samen naar Marseille zijn geweest wat toch een hele bijzondere ervaring was (ik heb nu een andere telefoon die niet meer "zomaar" piept). Verder wil ik je bedanken voor die keren dat ik even aan kwam kloppen om te praten over het werk, of andere dingen. Fons, jij ook bedankt dat je daar zo geduldig mee was, als ik weer eens ongevraagd binnen kwam vallen.

Onno, Elly, Birgitte, Monique en Fieke mijn speciale dank gaat naar jullie uit, omdat jullie na het vertrek van Astrid mijn dierwerk zijn gaan ondersteunen. Natuurlijk ook dank voor alle gezelligheid en interesse.

Op de vrijdag even ontspannen in de aesculaaf met Miranda en Twan (en Fieke, Mieke, Ruben …. en nog veel meer mensen). Ik hoop dat we dat weer een beetje in ere kunnen herstellen, want ik begin dat wel een beetje te missen.

Tim, we hebben samen toch nog mooi twee artikelen de deur uit kunnen doen. Bedankt voor je samenwerking en voor je gedeelde interesse in Fcγ receptoren. En niet te vergeten de gezellige congressen.

Erik (Vossie), ook jou wil ik bedanken voor de tips en adviezen bij de lay-out van mijn proefschrift. Verder was het gezamelijke congres in San Antonio erg leuk en gezellig afgelopen jaar. De volgende keer moeten we maar een vettere auto nemen om te scheuren op het strand.

Maar natuurlijk wil ik ook alle andere mensen die bij ons op het lab werken of hebben gewerkt bedanken. In een willekeurige volgorde: Henk, Nozomi, Cora, Erik, Liduine, Marianne, Linda, Marco, Mirjam, Laure, Isabel, bedankt voor jullie interesse en alle leuke dingen (dagjes uit, pool-wedstrijden, golf-clinics, sinterklaas, kerstvieringen etc.) de afgelopen jaren. Hierbij ook de buren van Biochemie bedankt.

Verder mogen de mensen van het dierenlab hier niet ontbreken. Zij hebben al die jaren mijn muizen verzorgd, waardoor mijn experimenten goed zijn verlopen.

Ook buiten het werk zijn mijn vrienden geïnteresseerd geweest in wat ik zo doe op het werk. Ondanks dat het bijna niet denkbaar is waar ik de hele dag mee vul, kan daar toch een boekje uit ontstaan. Kim en Marieke, bedankt dat jullie ook af en toe mijn gemekker wilden aanhoren (en fijn dat jullie dat ook weer snel konden relativeren).

Langs deze weg wil ik ook graag Jak bedanken voor zijn creatieve werk dat hij in mijn proefschrift heeft gestoken. Ondanks al je werk, heb je daar gelukkig tijd voor weten te vinden.

Naast mijn vrienden heeft de familie ook vaak geïnformeerd wat ik nou toch deed en of ik al iets had gevonden. Ik hoop bij deze dat mijn samenvatting een beetje duidelijk is, maar in ieder geval is dit stukje onderzoek afgerond.

Mijn Sis(sie) Anja. Ik vind het heel fijn dat je me bij zult gaan staan als mijn paranimf. Dank je wel voor alles wat je voor me doet en dat je altijd voor me klaar staat. Waarbij ik Simon natuurlijk ook niet mag vergeten!

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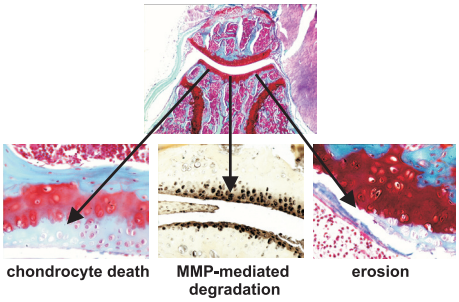
Pap en mam, voor jullie is dit boekje. Zonder jullie was dit er niet geweest. Jullie hebben mij altijd gestimuleerd om dat te gaan doen wat ik leuk vind en jullie hebben mij de kans gegeven om dat ook te kunnen doen. Bedankt voor al jullie steun, interesse en liefde al die jaren. Dit hoofdstuk is nu afgesloten, we gaan nu aan een nieuw beginnen!!!

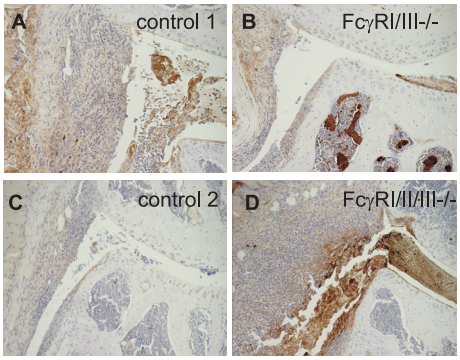
Colour Figures

Chapter 1

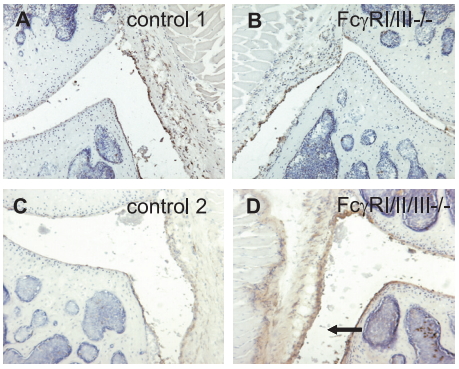


Chapter 1, Figure 2:
Parameters of severe cartilage destruction. Irreversible cartilage damage is characterized by chondrocyte death, MMP-mediated degradation, and erosion of the cartilage surface.

Chapter 3

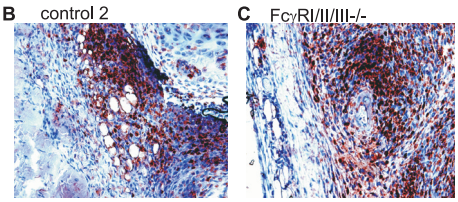


Chapter 3, Figure 4:
Presence of IgG-containing ICs, detected by immunolocalization, in knee joints of various KO mice, 7 days after induction of antigen-induced arthritis. Note that IgG is present in large amounts in arthritic FcγRI/II/III$^{-/-}$ knee joints when compared to their WT controls (D versus WT control C). No difference in amounts of IgG was found in arthritic knee joints of FcγRI/III$^{-/-}$ when compared to their controls (B versus WT control A). Original magnification, X 100.



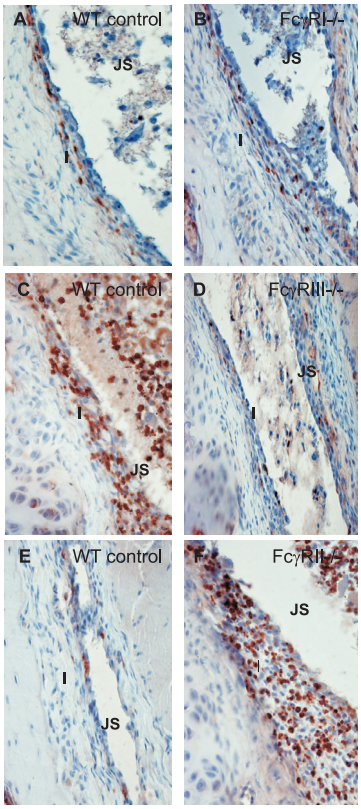
Chapter 3, Figure 5
Presence of heat-aggregated IgG detected by immunolocalization, 8 hours after injection in knee joints of various KO mice. Heat-aggregated rabbit IgG is less efficiently cleared (arrow) when injected in FcγRI/II/III$^{-/-}$ knee joints in comparison to WT controls 2 (D versus WT control C). No differences are found between knee joints of FcγRI/III$^{-/-}$ and their WT controls (B versus WT control A). Original magnification, X 100.

Chapter 3



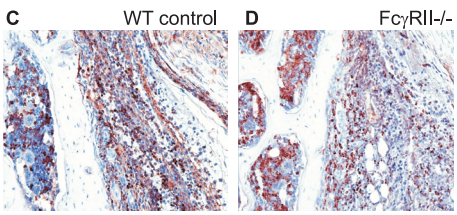
Chapter 3, Figure 7B and C:
Expression of activation markers MRP8 and 14 in synovial lining and joint cavity in FcγRI/II/III$^{-/-}$ and their WT controls at day 7 after AIA induction. Note the significantly higher expression of both MRP8 (A and C versus WT control B) and MRP14 (A). Data are the mean of 7 mice. Significance was tested using the Wilcoxon rank test (*, $P<0.05$). Original magnification, X 400.

Chapter 4

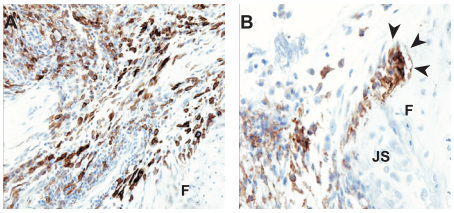


Chapter 4, Figure 2A-F:
Localization of myeloid-related protein 8 (MRP8) in the synovial lining and joint cavity at day 3 after ICA induction in FcγRI$^{-/-}$ (B), FcγRIII$^{-/-}$ (D), and FcγRII$^{-/-}$ (F) mice versus their wildtype controls (A, C, and E, respectively). Note the significantly lower MRP8 expression in FcγRIII$^{-/-}$ in the synovial lining (D versus C) and in the joint cavity (H) compared with WT controls, whereas similar expression was found in FcγRI$^{-/-}$ and controls (B versus A, and in G). Significantly higher MRP8 expression was found in FcγRII$^{-/-}$ versus controls both in the synovial lining (F versus E) and joint cavity (I). Bars show the mean and SD of 10 mice. * = P < 0.05, using the Wilcoxon rank test. I = intima lining (see Figure 1 for other definitions). (Original magnification X 400 in A-F).

Chapter 6

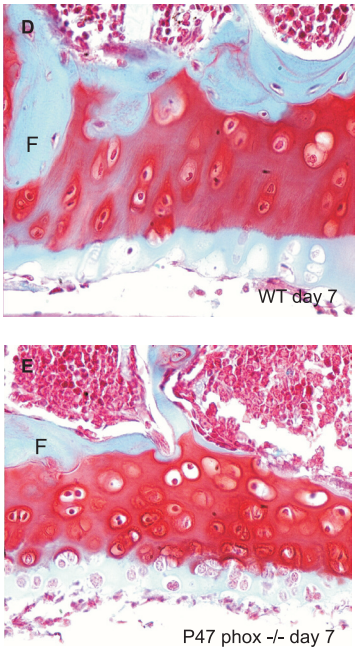


Chapter 6, Figure 2C and D:
Macrophages in the synovial lining (A, infiltrate) and in the joint cavity (B, exudate) and myeloid-related protein 8 (MRP8) in WT controls (C) and FcγRIII$^{-/-}$ mice (D), 3 days after ICA induction. Macrophages were detected using an antibody against F4/80. Note that after injection of AdIFN-γ, the percentage of macrophages was comparable in WT controls and FcγRIII$^{-/-}$ mice, whereas injection of PBS or AdeGFP resulted in significantly less macrophages in FcγRIII$^{-/-}$ mice. Values represent the mean ± SEM of 6 mice. Representative sections showing MRP8 localization which was comparable in arthritic knee joints of WT control (C) and FcγRIII$^{-/-}$ (D) mice. (Original magnification X 200.) * = P<0.05, Mann-Whitney U test.



Chapter 8, Figure 3:
Expression of the macrophage marker F4/80 7 days after induction of IFN-γ-stimulated ICA. Note that 70-80% of the infiltrated cells within the synovium consist of macrophages (A). F4/80 positive macrophages attached to the cartilage surface and were found in the lacunae of erosion pits (B, see arrows). Original magnification, X 400. F,femur; JS,joint space.

Chapter 8



Chapter 8, Figure 8 D and E:
Chondrocyte death in various cartilage layers of knee joints of P47phox$^{-/-}$ and their WT controls at day 3 after ICA induction (A) and at day 3 (B) and day 7 (C) after IFN-γ-stimulated ICA. Chondrocyte death was expressed as percentage of empty lacunae. Note that without IFN-γ, no chondrocyte is observed. At day 7 after induction of IFN-γ-stimulated arthritis, in WT controls chondrocyte death was clearly present, whereas in P47phox$^{-/-}$ chondrocyte death was completely absent (C and E versus control D). Significance was tested using the Wilcoxon rank test (*, $P<0.05$). MT, medial tibia; LT,lateral tibia; LF, lateral femur; MT, medial tibia; MF, medial femur; P, patella; F, femur. Original magnification, X 400.

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